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"BENEFICIAL EFFECTS OF DOCOSAHEXAENOIC ACID SUPPLEMENTATION IN VASCULAR FUNCTION OF ORCHIDECTOMIZED RATS"

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Memoria que presenta **Diva María Villalpando Grajeda** licenciada en Químico Bacteriólogo Parasitólogo para optar al grado de Doctor Internacional por la Universidad Autónoma De Madrid

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“Si he visto más lejos es porque estoy sentado sobre los hombros de gigantes”

Isaac Newton

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Abstract / Resumen

Abstract

The incidence of cardiovascular pathologies has been correlated with decreased levels of sex hormones. Beneficial effects of androgens on vascular function have been demonstrated by modulating the release and function of factors involved in vascular tone regulation. On the other hand, numerous studies support the cardioprotective properties of ω-3 polyunsaturated fatty acids (PUFAs), with docosahexaenoic acid (DHA) being one of the most studied.

Considering the pathophysiological effects observed as a consequence of the lack of sex hormones and the beneficial cardioprotective properties of the specific ω-3 PUFA, DHA, the current thesis investigated whether a diet supplemented with DHA could prevent the orchidectomy-induced alterations in vascular function from male rats. For this purpose, control and orchidectomized rats, fed with a standard diet, and orchidectomized fed with a supplemented diet with 5 % (w/w) DHA, were used to analyze: (1) the lipid profile, (2) the formation of cholesterol oxidation products (COPs), (3) redox status, and (4) inflammatory factors. In addition, responses to specific stimuli in isolated conductance and resistance vascular beds were also studied.

The results showed that the DHA supplemented diet prevented vascular dysfunction produced by the loss of gonadal function in male rats, since it restored the lipid profile negatively affected by the orchidectomy, prevented the orchidectomy-induced COPs increase, decreased hydrogen peroxide formation and increased antioxidant capacity in serum and aortic tissue. In addition, DHA-supplemented diet decreased the production of superoxide anion (increased by orchidectomy) in aorta and mesenteric artery, which may contribute to restore the NO production and bioavailability in these arteries. Also, DHA diet decreased prostanoid production in aorta and mesenteric arteries from orchidectomized rats. DHA also contributed to preserve vascular function, through the improvement of neurogenic responses in the mesenteric artery and in the participation of hyperpolarizing mechanisms in aorta. Overall, these results show that a DHA-supplemented diet exerted a cardioprotective effect in physiopathological conditions in which vascular dysfunction exists.

Resumen

La incidencia de patologías cardiovasculares se ha correlacionado con niveles disminuidos de hormonas sexuales. Así, se han descrito que los andrógenos ejercen efectos beneficiosos sobre la función vascular mediante la modulación de la liberación y función de factores implicados en la regulación del tono vascular. Por otro lado, numerosos estudios apoyan las propiedades cardioprotectoras de los ácidos grasos poliinsaturados ω-3, siendo el ácido docosahexaenoico (DHA) uno de los más estudiados.

Teniendo en cuenta los efectos fisiopatológicos observados como consecuencia de la falta de hormonas sexuales y las propiedades cardioprotectoras de los ácidos grasos ω-3, específicamente del DHA, en la presente tesis se investigó si una dieta suplementada con DHA podría prevenir las alteraciones inducidas por la orquidectomía en la función vascular de ratas macho. Para este propósito, se utilizaron ratas control y orquidectomizadas alimentadas con una dieta estándar y orquidectomizadas alimentadas con una dieta suplementada con DHA, 5% (p/p), para analizar: (1) el perfil lipídico, (2) la formación de productos de oxidación del colesterol (COPs), (3) estado redox y (4) factores inflamatorios. Además, se estudiaron las respuestas a estímulos específicos en lechos vasculares de conductancia y de resistencia.

Los resultados mostraron que la dieta suplementada con DHA previene la disfunción vascular producida por la pérdida de la función gonadal en ratas macho, ya que restauró el perfil lipídico, afectado negativamente por la orquidectomía, impidió el aumento de COPs inducido por orquidectomía y disminuyó la formación de peróxido de hidrógeno, a la vez que aumentó la capacidad antioxidante (ejerciendo así propiedades antioxidantes) en suero y tejido aórtico. Además, la dieta suplementada con DHA disminuyó la producción del anión superóxido (aumentado por la orquidectomía) en aorta y arteria mesentérica, lo que probablemente contribuyó a restaurar la producción y biodisponibilidad del óxido nítrico en estas arterias. Además, el DHA ejerció propiedades antiinflamatorias, ya que disminuyó la producción de prostanoides en aorta y arteria mesentérica de ratas orquectomizadas. El DHA también contribuyó a preservar la función vascular, participando en la mejora de las respuestas neurogénicas de la arteria mesentérica y en la participación de mecanismos hiperpolarizantes en la aorta. En general, estos resultados muestran que una dieta suplementada con DHA ejerce un efecto cardioprotector en condiciones fisiopatológicas en las que existe disfunción vascular.

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List of abbreviations

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$[Ca^{2+}]_i$ Intracellular calcium concentration

25-OH 25-Hydroxycholesterol

27-OH 27-Hydroxycholesterol

5,6α-E 5,6α-epoxycholesterol

5,6β-E 5,6 β-epoxycholesterol

5-HT Serotonine

7-keto 7-ketocholesterol

7α-OH 7α-Hydroxycholesterol

7β-OH 7β-Hydroxycholesterol

AA Arachidonic acid

AC Adenylate cyclase

ACh Acetylcholine

ADP Adenosine diphosphate

ALA α-Linoleic acid

Ang-II Angiotensin II

ANOVA Analysis of variance

ATP Adenosine triphosphate

BH_4 cofactor tetrahydrobiopterin

BK Bradykinin

Ca^{2+} Calcium

cAMP Cyclic adenosine monophosphate

cGMP Cyclic guanosine monophosphate

CGRP Calcitonin gene-related peptide

CO Carbon monoxide

COPs Cholesterol oxidation products

COX Cyclooxigenase

CRLR Calcitonin receptor-like receptor

CVD Cardiovascular disease

CYP450 Cytochrome P450

DAF Diaminofluorescein

DAG Diacylglycerol

DHA Docosahexaenoic acid

EDHF Endothelium-derived hyperpolarizing factor

EETS eicosatrienoic acids

EFS Electrical field stimulation

eNOS Endotelial nitric oxide synthase

EPA Eicosapentaenoic acid

ET-1 Endothelin-1

GTP Guanosine triphosphate

H^+ Hydrogen

H_2O_2 Hydrogen peroxide

HDL High density lipoprotein

HE Hydroethidine

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IL-6 Interleukin 6

iNOS Inducible nitric oxide synthase

IP_3 Inositol trisphosphate

K^+ Potassium

K_{2P} two pore domain potassium channel

K_{ATP} ATP-dependent potassium channel

K_{Ca} Calcium-dependent potassium channel

KHS Krebs-Henseleit solution

K_{IR} Inward rectifier potassium channel

K_V voltage gated potassium channel

LA Linoleic acid

LDL Low density lipoprotein

L-NAME Nω-Nitro-L-arginine methyl ester

L-NMMA L-NG-monomethyl arginine citrate

monophosphate

NA Noradrenaline

NADPH Nicotinamide adenine dinucleotide phosphate

nNOS Neuronal nitric oxide synthase

NO Nitric oxide

NOS Nitric oxide synthase

NPY Neuropeptide Y

O_2 Oxygen

O_2^- Superoxide anion

OH^- Hydroxyl radical

$ONOO^-$ Peroxynitrite

ORAC Oxygen radical absorbance capacity

PGD_2 Prostaglandin D_2

PGE_2 Prostaglandin E_2

$PGF_{2\alpha}$ Prostaglandin $F_{2\alpha}$

PGG_2 Prostaglandin G_2

PGH_2 Prostaglandin H_2

PGI_2 Prostaglandin I_2

PIP_2 Phosphatidyl-inositol

PKA Protein kinase A

PKC Protein kinase C

PKG Protein kinase G

PLA_2 Phospholipase A_2

PLC Phospholipase C

RAMP receptor activity-modifying protein

ROS Reactive oxygen species

SEM Standard error of the media

Ser Serine

$_sGC$ soluble guanylate cyclase

SNP Sodium nitroprusside

SOD Superoxide dismutase

Thr Threonine

TNF-α Tumor necrosis factor alpha

TRP transient receptor potential

TXA_2 Thromboxane A_2

TXB_2 Thromboxane B_2

Tyr Tyrosine

VIP Vasoactive intestinal polypeptide

VOOC voltage operated calcium channels

VSMCs vascular smooth muscle cells

WHO World Health Organization

Introduction

Cardiovascular diseases

Cardiovascular disease is a collective term that constitutes a group of disorders of the heart and blood vessels. According to the World Health Organization (WHO) cardiovascular diseases (CVD) are the leading cause of death worldwide [1]. In the spanish population, one of every three deaths is caused by CVD, age-adjusted mortality rates for these pathologies have been decreasing since 1975 by 3.1% per year [2]. In contrast, rates of hospital morbidity due to CVD have almost tripled in Spain during this period due to the increase in obesity and diabetes incidence, coupled with increased patient survival and aging in the general population, although it seems to have stabilized since 2002 [3]. The CVDs with the highest incidence are cerebrovascular disease in women and ischemic heart disease in men [2].

CVD occurrence is determined by multiple factors and their interactions. It is well established from the Framingham study, the importance of several modifiable CVD risk factors, among which we can highlight hypertension, hypercholesterolemia, smoking and diabetes mellitus and others, such as hypertriglyceridemia, obesity and sedentary lifestyle that also play a relevant role [4–6]. However, some risk factors, including age, gender or family history/genetic predisposition, are immutable [7]. Additionally, cultural and psychosocial factors, e.g. education, economic and marital status, also access to health care, contribute to the development of CVDs [1]. Therefore, the combination of lifestyle and the risk factors associated with the development of CVD is related to its higher incidence. Thus, the control of these CVD risk factors and the modification in the habits of life can delay or even sometimes avoid the appearance of these conditions. Additionally, the effect of sex hormones seems to influence the incidence of CVD, which will be described in the "*Sex hormones and regulation of vascular tone"* section.

Nowadays, there are numerous cardiovascular risk prediction biomarkers. It is well stablished that a standard lipid profile including total cholesterol, LDL (low density lipoprotein) cholesterol, HDL (high density lipoprotein) cholesterol and triglycerides, is recommended to predict the risk to develop a CVD. Also, glucose and hormones levels can be correlated with CVD incidence. In addition, atherosclerosis is a common pathophysiological condition present in several CVD, since it is a multifactorial disease whose onset influences various CVD-risk factors (hypertension, hyperlipidemia, etc.), that interact synergistically and tend to be associated in some individuals. Moreover, various cardiovascular risk prediction models have been updated by incorporating traditional risk factors and molecular, immunological, genetic,

imaging, and biophysical factors for more authentic and reliable estimation of cardiovascular risk [8].

Cholesterol as a cardiovascular risk factor

Cholesterol is one of the major components of cell membranes and is involved as a substrate in the synthesis of steroid hormones and bile acids. To perform their functions, all cells require cholesterol, obtained by de *novo* biosynthesis, or by taking it from the lipoproteins present in the plasma. Cholesterol is vital in the body, but when it is found in excess dyslipidemia may appear. These alterations can be of dietary or genetic origin, also the existence of alterations in the synthesis of cholesterol or in its transporting lipoproteins may be involved [9].

In hypercholesterolemia, cholesterol accumulates in the lipid bilayers from different types of cells, including erythrocytes, platelets, leukocytes, polymorphonuclear cells, and endothelial cells. These cholesterol molecules are largely susceptible to oxidation, leading to the formation of cholesterol oxidation products (COPs) or also known as oxysterols [10]. The oxidation of this unsaponificable molecule affects the properties and the dynamics of the lipid bilayers by causing minor changes in the chemical structure of cholesterol, which in turn influences cell signaling [11–13].

In biological systems the transformation of cholesterol into its oxidized derivatives occurs trough non-enzymatic or enzymatic reactions. Generally, oxysterols differ from cholesterol by one or several additional polar groups, which typically include a hydroxyl, keto, hydroperoxy, epoxy, or carboxyl group. In **Figure 1** the main biologically relevant oxysterols are represented.

Non-enzymatic cholesterol oxidation, also known as cholesterol auto-oxidation is given by its reaction with reactive oxygen species (ROS), which are physiologically present in the organism [14]. In this respect, the oxidation of cholesterol is similar to that occurred in other lipids, also prone to attacks by ROS [15,16].

As well, cholesterol can be oxidized through to the action of several enzymes [17,18] mostly related to the cytochrome P450 family. Among them, CYP7A1 and CYP27A1, take part in the synthesis of 7α-hydroxycholesterol (7α-OH) and 27- hydroxycholesterol (27-OH), occurred predominantly in the liver [19]. Cholesterol 25- hydroxylase, which is a non heme-iron-containing enzyme present in most tissues, catalyzes the formation of 25 hydroxycholesterol (25-OH) [18].

In addition, exogenous oxysterols can be absorbed from dietary sources [20] and enter circulation[15,21].

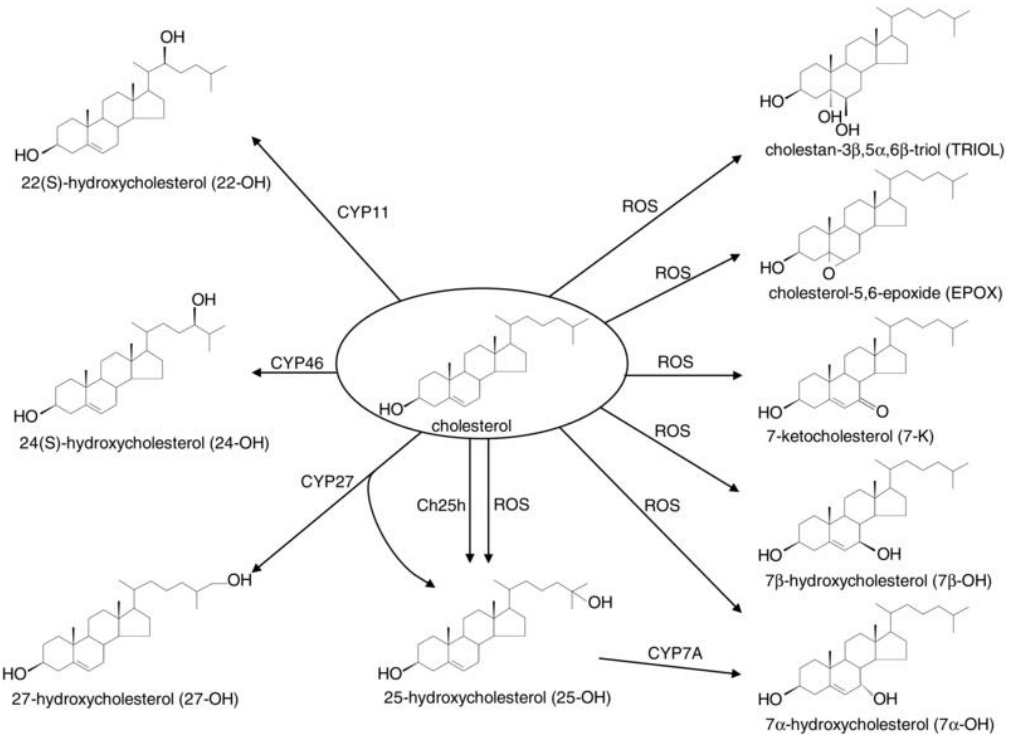

Figure 1. Chemical structures of the main oxysterols formed enzymatic and non-enzymatic pathway [22]. Cholesterol is predominantly modified at carbon 7, leading to the formation of 7α-OH and 7β-hydroxycholesterol (7β-OH) and 7-ketocholesterol (7-keto), recognized as the main products of cholesterol autoxidation. Cholesterol is also commonly modified in carbons 5 and 6 to form 5β,6β-epoxycholesterol (5β-E), 5α,6α-epoxycholesterol (5α-E) and 5α,6β-dihydroxycholesterol. In a lesser extent, the oxidation of the side chain generates monohydroperoxides in positions 20, 22, 24, 25 and 26, leading to the formation of hydroxyl derivatives, comprising oxysterols of biomedical interest, although they are generally considered to not represent significant products of the autoxidation of cholesterol [14,15,23].

Lipid peroxidation induces disturbance of fine structure, alteration of integrity, fluidity, and permeability, and functional loss of biomembranes. The growing evidence implicates the accumulation of cholesterol metabolism end products as mediators of lipid metabolism, inflammation, potent oxidative stress inducers, cell death, and autophagy on different cell types [22]. In addition, the reactive carbonyl compounds, the secondary products of oxysterols, modify proteins and DNA bases, showing mutagenic and carcinogenic properties [24]. In fact, many studies report increased levels of oxysterols products in biological tissues and fluids of patients in pathologies including atherosclerosis, cardiovascular diseases, cancer, neurological disorders [25,26], metabolic syndrome, osteoporosis, macular degeneration, inflammatory bowel diseases, diabetes, kidney failure, ethanol intoxication [27] and in

physiological conditions characterized by altered cholesterol uptake and/or metabolism like aging [28].

General structure of the vascular wall

Blood vessels withstand the pressure of circulating blood trough the body, distribution of nutrients, gases, removal of waste products and thermoregulation, providing the main link between the heart and tissues. The arterial wall contributes to the maintenance of vascular homeostasis, since it responds actively to a variety of stimuli. The mechanisms involved in the regulation of vascular tone are diverse, in which endothelial, muscular, nervous and hormonal factors are involved [29].

The vascular wall consists of three layers; the intima (inner layer), the tunica media (middle layer) and the adventitia (outer layer) (**Figure 2**). Vasomotor control, as well as the vascular structure, are influenced synergistically by each of the layers, in which the final effect is the result of the interconnected participation of the three arterial layers [30].

The *endothelium* is a monolayer of cells that delimitates the inside of the vascular wall, which is composed by the intima layer along with its connective tissue. The endothelium forms a barrier between the circulatory content and the surrounding vascular tissue. It regulates vascular tone by affecting the balance between dilatation and constriction of vascular smooth muscle cells (VSMCs). It also controls a variety of other intravascular processes such as proliferation and migration of VSMCs, leukocyte and platelet adhesion and aggregation, as well as the balance between thrombogenesis and fibrinolysis [31]. The endothelial function refers to the homeostasis of this set of physiological mechanisms, which is maintained by the synthesis and release of multiple vasoactive mediators. Among the vasodilator and anti-atherogenic factors participating in this function is the nitric oxide (NO), the prostacyclin (PGI_2) and the endothelium derived-hyperpolarizing factor (EDHF). On the other hand, the vasoconstricting and pro-atherogenic substances of this function involve prostanoids, such as thromboxane A_2 (TXA_2), ROS, angiotensin II (Ang II) and endothelin-1 (ET-1) [32]. It is known that endothelial cells are sensitive to hemodynamic changes, which combined with the action of the released factors maintain vascular homeostasis.

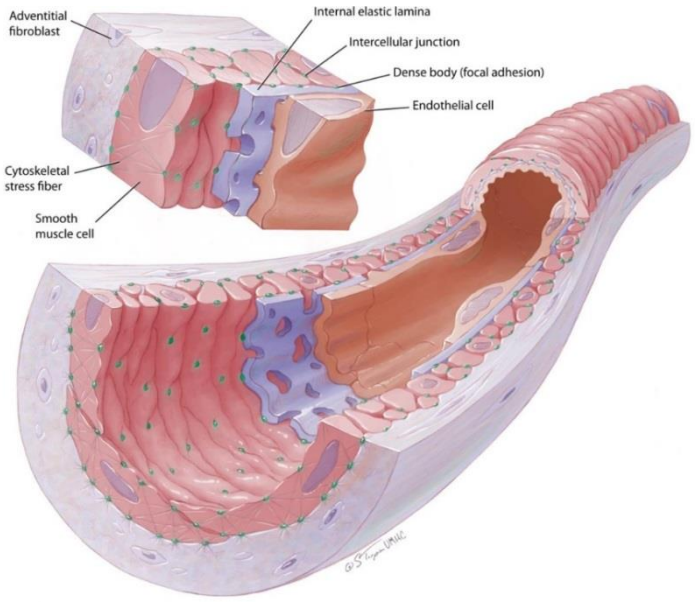

Figure 2. General structure of the arterial wall. Adapted from Martinez-Lemus [33]. The arterial wall is a heterogeneous, three-layered structure composed of an intima, media, and adventitia. Each layer displays specific histological, biochemical, and functional characteristics, to contribute in maintenance of vascular homeostasis and vascular response regulation to stress or injury.

Endothelial dysfunction is an imbalance between vasoconstrictor and vasodilator mediators [34], resulting in the loss of selective barrier function, a greater tendency for platelet aggregation and thrombosis, increased leukocyte adhesion, vasoconstriction, as well as migration of VSMCs into the inner layer of the vessels. Endothelial dysfunction is probably the first step towards hypertension and atherosclerosis and it is associated with numerous CVD. Also, is closely related to other pathologies such as hyperlipidemia and diabetes, smoking, obesity, age or sedentary lifestyle [35].

The *tunica media* is the middle layer and primarily consists in a very marked layer of VSMCs fibers with reticular fibers associated therewith and a small number of individual elastic fibers. Vascular smooth muscle constitutes the major part of this layer in blood vessels and plays an important role in the regulation of vascular resistance and blood pressure, and its deregulation can lead to vascular diseases, including hypertension and coronary artery disease.

Vascular muscle cells are fusiform elongated cells with an enlarged nucleus, and contain actin and myosin myofibrils, responsible for contraction due to the displacement of actin on myosin filaments. The contractile process is driven by the increase of cytoplasmic concentration of Ca^{2+} ($[Ca^{2+}]_i$) and/or by activation of different protein kinases [36–38] Also, VSMCs are able to produce different vasoactive substances, including prostanoids, growth factors, ROS, cytokines, among others.

The *tunica adventitia* is the outermost layer of the vessel wall, surrounding the tunica media. The adventitia is predominantly made up by extracellular matrix (type I collagen and elastic fibers), nutrient vessels (*vasa vasorum*), autonomic nerves (*nervi vasorum*) and adipose tissue [39,40]. Fibroblasts and numerous macrophages are also present in this layer. The tunica adventitia helps to anchor the vessel to the surrounding tissue. The adventitia actively participates in the regulation of vascular tone, since it is a source of fatty acids, adipokines and ROS, which affect vascular smooth muscle function [41].

The nerve endings that reach the adventitial tunica have great importance in the control of vasomotor responses. Between the nerve endings and the vascular cells, chemical communication is established through the synaptic cleft. This type of communication is characterized by the release of neurotransmitters from the nerve endings, and by the interaction of these neurotransmitters with specific receptors located in the postsynaptic cell.

A brief review of the main factors involved in the regulation of vascular function will be presented below.

Nitric oxide

NO is a small gas molecule that plays a key role in every system of the organism. Given the importance of this molecule in the regulation of vascular tone it has been widely studied, and the mechanisms related to its synthesis, release, metabolism and the mechanism of action are very well known [32]. The NO biosynthesis is catalyzed by three different isoforms of the NO synthase enzyme (NOS) of which there are two constitutive isoforms called endothelial NOS (eNOS), present mainly in the endothelium, endocardium and myocardium [42] and neuronal NOS (nNOS) present in the central and peripheral nervous system and also in cardiomyocytes [43], which are Ca^{2+}-calmodulin dependent. The third inducible isoform, called inducible NOS (iNOS) is located in different cell types, is Ca^{2+} and calmodulin independent, and can be induced by cytokines or bacterial endotoxins [44].

In the synthesis of NO, NOS hydroxylates the L-arginine to N^{ω}-hidroxy-L-arginine (which remains largely bound to the enzyme). In a second step, NOS oxidizes N^{ω}-hidroxy-L-arginine to L-citruline and NO using nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor [45]. The three isoforms of the NOS enzyme go through these two stages for the synthesis of NO. eNOS can also be stimulated pharmacologically, by endothelium-dependent vasoactive substances, which can influence the enzyme detachment from caveolin by releasing Ca^{2+} from the endoplasmic reticulum. Examples of these substances include bradykinin (BK), acetylcholine (ACh), adenosine triphosphate (ATP), adenosine diphosphate

(ADP), substance P and thrombin [46]. The enzyme can be inhibited by a wide variety of substances, e.g. L-arginine analogs, like the N^{ω}-monomethyl-L-arginine (L-NMMA) or N^{ω}-nitro-L-arginine methil esther (L-NAME).

NO derived from eNOS is importantly involved in the maintenance of basal vascular tone [47], but may also confer vasoprotection, since it is a homeostatic regulator of essential cardiovascular functions. NO causes relaxation in blood vessels by stimulating soluble guanylyl cyclase (sGC) and increasing cyclic guanosine monophosphate (cGMP) in smooth muscle cells (**Figure 3**) [48,49].

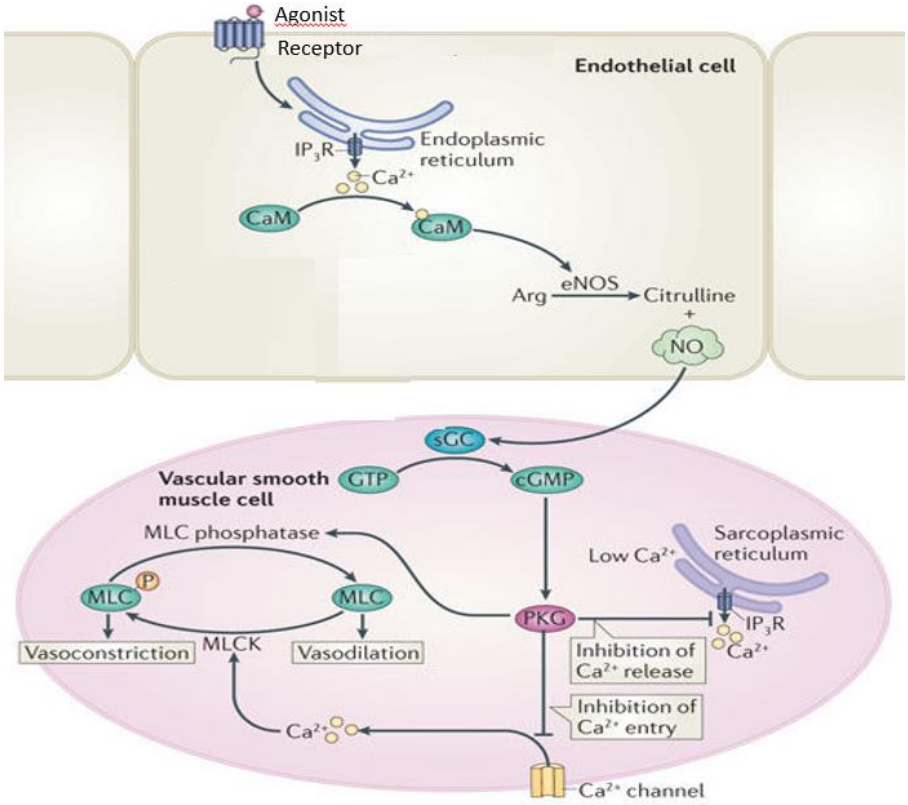

Figure 3. Schematic representation of VSMc relaxation by NO. Adapted from Paul & Snyder [50]. NO diffuses rapidly through tissues such as VSMCs; NO causes a conformational change in the sGC to increase activity of this enzyme [48,51]. The sGC converts the guanosine triphosphate (GTP) into cGMP, which in turn activates the protein kinase G (PKG). Phosphorylation of the Ser and Thr residues from the PKG activates or inhibits the function of different proteins to decrease $[Ca^{2+}]_i$ and produces relaxation in the fiber through different mechanisms, such as the activation of the Ca^{2+}-ATPase of the sarcoplasmic reticulum by introducing Ca^{2+} into the reticulum [52], the regulation in the phosphorylation and activation of the Ca^{2+} -ATPase of the plasma membrane, releasing Ca^{2+} to the outside of the cell [53], closure of the plasma membrane voltage-dependent Ca^{2+} channels [54], the inhibition of Ca^{2+} release from the reticulum induced by phosphorylation of inositol trisphosphate (IP_3) [55], activation of Na^+/K^+ ATPase [56], activation of Ca^{2+} -dependent K^+ channels (Robertson *et al.*, 1993), stimulation of Na^+/Ca^{2+} exchanger [57]. In addition, it has been reported that NO can hyperpolarize the smooth muscle cell membrane and thus cause vasodilation by direct activation of K^+ channels [58]**.**

eNOS can also induce a long-lasting release of NO in an intracellular Ca^{2+}-independent manner. In vascular endothelium, the best stimulus to activate eNOS for the generation and release of NO, is the hemodynamic stimuli exerted on the luminal surface of endothelial cells by the streaming blood (shear stress) [59]. This activation of eNOS is mediated by phosphorylation in various residues of serine (Ser), threonine (Thr) and tyrosine (Tyr) through protein kinases [60]. Also, NO is a potent inhibitor of platelet aggregation and adhesion to the vascular wall [61,62], which in turn exerts anti-proliferative mechanisms by avoiding the release of platelet-derived growth factors. eNOS is also essential in adaptive vascular remodeling in chronic changes of flow [63] and controls the expression of genes involved in atherogenesis [64], participates in mRNA translation [65] and produces post-translational protein modification [66].

The NO produced by nNOS in the nitrergic nerves acts as a neurotransmitter that stimulates the NO-responsive GC in its effector cells, thus decreasing the tone of various types of smooth muscle including blood vessels [67,68].

Inducible NOS is not commonly expressed, but can be induced by cytokines and other agents (lipopolysaccharides) in almost any cell type. Unlike eNOS and nNOS, iNOS is constantly active once expressed, and is not regulated by $[Ca^{2+}]_i$. However, the expression of the iNOS and the NO generated by this enzyme mediate various symptoms in inflammatory diseases [69]; the major cytotoxic effect on phagocytes due to the production of large amounts of NO, leading to DNA breakage and fragmentation in its target cells (i. e. microorganisms and tumor cells) [70,71]. In addition, inducible NOS-derived NO is the main mediator of vasodilation and the drop in blood pressure observed in septic Shock [72]. The levels of NO released from iNOS is much higher compared to the other two NOS isoforms [73].

Endothelium-dependent hyperpolarizing factor

The endothelium also releases a relaxation-inducer substance in vessels by hyperpolarizing the muscle membrane, called the endothelium-dependent hyperpolarizing factor (EDHF) [74]. Currently, EDHF is attributed to several completely different mechanisms, not just one, ranging from K^+ [75] to H_2O_2 [34] (**Figure 4**). The activity of EDHF is not mediated by NO or cyclooxygenase (COX) -derived prostanoids, but it is blocked, at least partially, by inhibitors of K^+ channels. Endothelium-dependent hyperpolarization is substantially due to the opening of the K^+ channels in the VSMCs, determining the K^+ flux and consequent hyperpolarization of the cell membrane. This, in turn, induces a decrease in the influx of Ca^{2+}, which results in vasorelaxation [76].

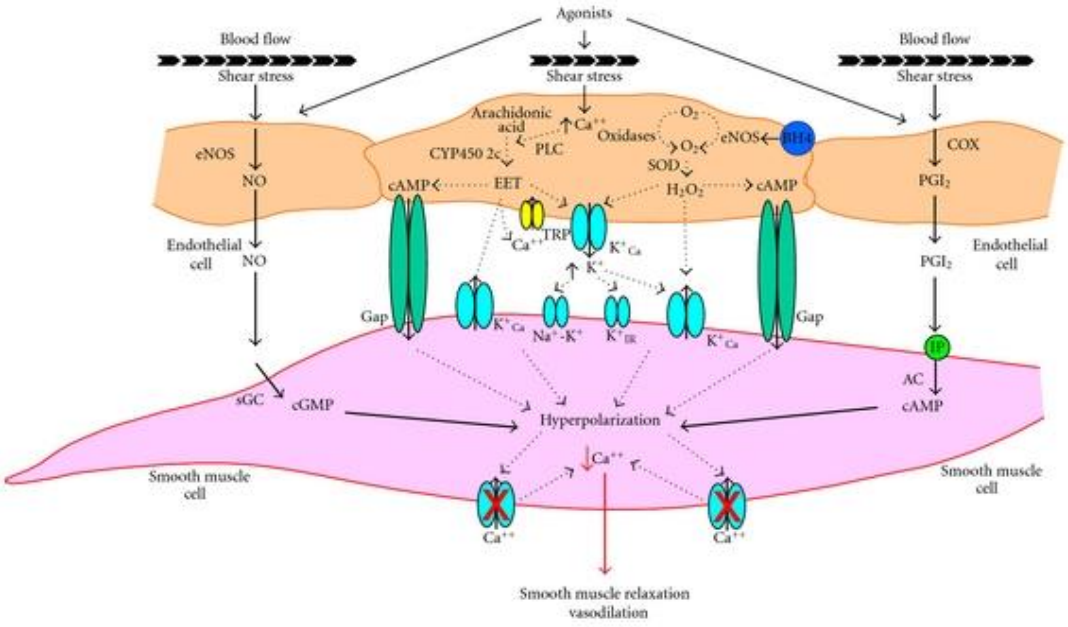

Figure 4. Schematic representation of the mechanisms likely involved in endothelium-derived hyperpolarization. Mechanisms for endothelial cell mediated relaxation. Agonist (bradykinin/acetylcholine/substance P) or shear stress increases the activity of eNOS and COX, providing NO and PGI_2-mediated dilation. There are multiple potential EDHF pathways. $[Ca^{+2}]_i$ activates PLA_2 to produce AA. Its metabolism by cytochrome P450 2C (CYP4502c) generates eicosatrienoic acids (EETs) that can stimulate calcium-dependent potassium (K_{Ca}) channels in endothelial and smooth muscle cells. EETs may also directly activate gap junctions (Gap). EETs may also act in an autocrine manner on endothelial cells by activating transient receptor potential (TRP) V4 channels, which promote Ca^{+2} influx further increasing the calcium concentration and activating K_{Ca} channels to cause hyperpolarization and release of K^+ ions into the subendothelial space. The increase in potassium in the interstitium may activate K_{Ca} $^+$channels, inwardly rectifying potassium channels (K_{IR} $^+$), or the Na^+-K^+ pump on smooth muscle cells and cause hyperpolarization. Smooth muscle hyperpolarization in turn results in relaxation by closing voltage-gated channels leading to a fall in Ca^{+2} concentration and subsequent vasodilation. The action of eNOS (with cofactor tetrahydrobiopterin [BH4]) and oxidases on oxygen (O_2) produces the reactive oxygen species superoxide (O_2^-). Hydrogen peroxide (H_2O_2) generated by dismutation of superoxide anions by SOD can also cause hyperpolarization by activating endothelial and smooth muscle K_{Ca} channels or by gap junctions. Adapted from Ozkor *et al.* [77].

The endothelium and smooth muscle express a variety of different K^+ channels, Ca^{2+} and ATP-dependent channels (K_{Ca} and K_{ATP} respectively), voltage-dependent (K_V), two pore domain (K_{2P}) and input rectifiers (K_{IR}). Their contribution to the numerous mechanisms of endothelium-dependent relaxation and smooth muscle are closely related to their electrophysiological properties and mechanisms of activation within the vascular wall. Therefore, K^+ channels play an important role in the regulation of blood pressure. Mechanisms of action of EDHF include: (a) synthesis of cytochrome P450 (CYP450) labile metabolites, a family of epoxides, (b) transmission of endothelial cell hyperpolarization to the vascular

smooth muscle via gap junctions, and (c) K^+ released from the endothelial cells via K_{Ca} or K_{ATP} channels induces smooth muscle hyperpolarization by activating K_{Ca} channels and/or Na^+-K^+-ATPase on vascular smooth muscle cells. Hydrogen peroxide (H_2O_2) is involved in K_{Ca} channels activation, therefore considered as an EDHF. Also, some non-NO gaseous agents, such as carbon monoxide (CO) and hydrogen sulphide (H_2S) which might act as autocrine and/or paracrine agents to produce hyperpolarization [78,79]. The contribution of EDHF to endothelium-dependent relaxation varies depending on the artery studied. This factor plays a fundamental role in resistance vessels, thus appears to play an important role in local regulation of peripheral vascular resistance [80]. Additionally, their participation is modified in pathophysiological situations in which NO bioavailability is diminished, acting as a reserve system for endothelial vasodilator factors, even in conductance vessels [75,81,82].

Prostanoids

Prostanoids are synthesized by COX from arachidonic acid (AA), a 20-carbon unsaturated fatty acid. Prostanoids biosynthesis begins with the release of AA from membrane phospholipids by the action of phospholipase A_2 (PLA_2) or the diacylglycerol (DAG) [83]. AA is converted to prostaglandin G_2 (PGG_2), which is subsequently peroxidized to prostaglandin H_2 (PGH_2). The catalysis of these reactions is carried out by the COX, adding two molecules of O_2 to AA for the generation of PGG_2. Subsequently, the peroxidase activity catalyzes the reduction of PGG_2 to PGH_2 (**Figure 5**) [84].

There are two isoforms of the enzyme cyclooxygenase, COX-1 and COX-2, encoded by two different genes, and it is associated with the luminal side of the endoplasmic reticulum and the nuclear membrane [85]. Both isoforms are expressed in endothelial cells and in VSMCs [86]. COX-1 is located on the membrane of the endoplasmic reticulum, constitutively expressed in most tissues [87]. It is the predominant isoform under static conditions or under chronic shear stress [88,89]. Also, plays a very important role in different physiological functions, including platelet aggregation and renal function [90]. The COX-2 isoform is found in the nuclear membrane at very low levels. However, it is immediately induced by mitogenic and pro-inflammatory stimuli, such as cytokines (interleukin-1β) and endotoxins (lipopolysaccharides) in endothelial cells, smooth muscle cells and fibroblasts [91–93]. However, COX-2 is also expressed constitutively in different cell types, including endothelial cells, in which its expression is also regulated by mechanical stress [94]. Also, it has been described an enzymatically active COX-1 splicing variant called COX-3, expressed in the heart and cerebral cortex, but the regulation of its transcription seems similar to that of COX-1 [95].

PGH_2 is the substrate of the different synthases for the production of different prostanoids (**Figure 5**). The bioactive prostanoids are the prostaglandin (PG) E_2, $PGF_{2\alpha}$, PGD_2, PGI_2, and thromboxane A_2 (TXA_2) [86]. Prostanoids interact with their specific receptors, which are coupled to heterotrimeric G proteins that modulate second messengers, such as cAMP or IP_3 at the intracellular level. However, the effect of each prostanoid will depend on the cell type, physiological situation, concentration or whether it binds to another receptor from the same family of proteins, which may result in an opposite effect [96].

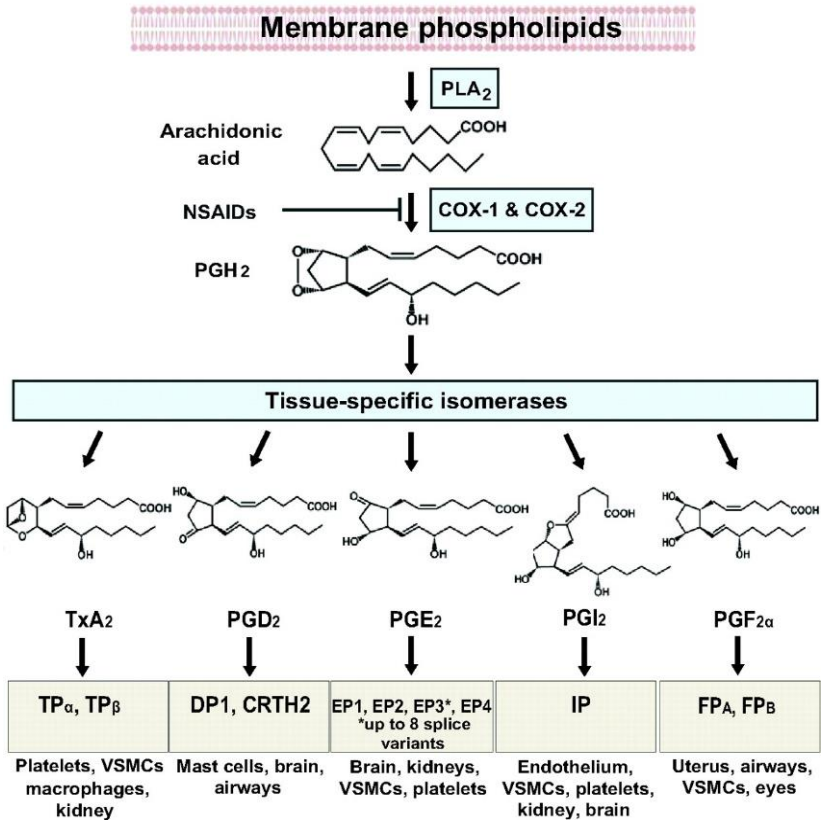

Figure 5. **Arachidonic acid metabolism towards specific prostanoids**. Formation of prostanoids from AA by the action of COX-1/2. The specific effects of each prostanoid occur through the activation of the specific receptors (IP, TP, DP, EP and FP).

PGE_2 is the predominant product of COX in microvascular endothelial cells [97] and in general is the most abundant prostaglandin in the human body. This molecule exerts reproduction, neuronal, metabolic and immune functions [98]. Among its physiological effects, comprises the regulation of blood pressure through VSMCs relaxation and contraction, depending on the activated receptor. These effects can be attributed to the existence of four receptor subtypes, EP1, EP2, EP3 and EP4, which are coupled to different signaling pathways [96,98,99]. Activation of EP1 is associated with contraction [100,101]. In contrast, activation of EP2 and EP4 receptors leads to vasodilation [102]. PGE_2 activates platelets through EP3 and

possibly EP1, but at high concentrations it may inhibit its aggregation by stimulating the IP and TP receptors [103].

The $PGF_{2\alpha}$ isoforms are synthesized from PGH_2, and also from PGD_2 and PGE_2 [104,105]. $PGF_{2\alpha}$ interacts with the FP receptor, which generally causes an increase in $[Ca^{2+}]_i$ and vasoconstriction. The $PGF_{2\alpha}$-induced activation of TP receptors has been involved in the development of cardiac hypertrophy [96,106,107]. Additionally, FP receptor stimulation can be expressed in endothelial cells and induce endothelial NO release [108].

Prostacyclin is an unstable substance formed by prostacyclin synthase, which is highly expressed in endothelial cells [101]. It is also found in cardiac and VSMCs, among other cell types [109]. It is a potent platelet inhibitor, which prevents its aggregation and adhesion to the surface of endothelial cells. Prostacyclin is the preferential ligand of IP receptors, producing vasodilatory effect [83] by reducing intracellular Ca^{2+} in muscle cells by the action of cAMP after activation of adenylate cyclase (AC). cAMP induces the phosphorylation of protein kinase A (PKA) producing the inhibition of Ca^{2+}-calmodulin dependent contractile mechanisms [110] by inhibiting the release of Ca^{2+} from the reticulum by the phosphorylation of the IP_3 receptor. Therefore, in numerous of vascular beds, PGI_2 may act not only as an endothelium-derived relaxing factor, but also as a hyperpolarizing substance derived from the endothelium, since it activates K^+ channels [34,111]. In addition, prostacyclin is also a potent anti-proliferative agent in vascular smooth muscle cells, reduces oxidative stress and prevents cell adhesion to the vascular wall [109]. It has been shown that PGI_2 levels are increased in patients with atherosclerosis [112], and under experimental conditions of hypertension [82,113]. The vasodilatory effect induced by low concentrations of PGI_2 turns into vasoconstrictor due to the increased production, whereas at high concentrations the effect is vasoconstrictor [83,113].

TXA_2 is synthesized by the action of the enzyme thromboxane synthase and it is rapidly metabolized into TXB_2. In the cardiovascular system, TXA_2 is derived from COX-1, which is synthesized in endothelial and smooth muscle cells [94], but it can also be produced by COX-2 in the vascular wall [114]. TXA_2, like all prostanoids, exerts its actions through its specific receptor, called TP, of which two different isoforms called α and β are known, distinguished from each other at the carboxy- end located inside the cell [115]. The release of TXA_2 and other vasoconstrictor PGs can be stimulated by different vasoconstricting agents such as ET-1, serotonin (5-HT), noradrenaline (NA) and Ang-II, as well as vasodilator agents, like ACh, and other substances such as nicotine and ionophore A23187 [116,117]. Thus, TXA_2 can participate in the endothelial dysfunction associated with cardiovascular disease [118]. TXA_2 activates its specific receptor, TP, which is functionally coupled to a G protein. The activation of these components inhibits the AC, leading to a decrease in cAMP levels [115]. Activation of TP

receptors also induces the activation of phospho lipase C (PLC). PLC catalyzes the transformation of phosphatidylinositol-4,5-bisphosphate (PIP_2) into DAG and IP_3, resulting in the release of stored Ca^{2+} and PKC activation respectively, leading to the contraction of the smooth muscle cells [119]. In addition to the activation of these signaling pathways, TXA_2 induces platelet aggregation and VSMCs contraction, hypertrophy and cell proliferation [120]. TXA_2 is also related to hypertension, atherosclerosis, myocardial infarction and in general to the development of CVD [121].

Reactive oxygen species

Reactive oxygen species (ROS), such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (^{-}OH), are generated by the cell normal metabolism. ROS have a significant role as intracellular signaling molecules and as paracrine messengers in physiological and pathological processes. Both the endothelial cells [122–125] and smooth muscle cells [123,126] generate significant amounts of ROS, spontaneously or in response to receptor-mediated or non-receptor-mediated stimuli. Under normal conditions, there is a balance between the formation and removal of ROS by antioxidants. However, an imbalance between pro-oxidants and antioxidants results in oxidative stress, which has been associated with multiple pathologies, including CVD [127]. In the vascular system, ROS can modulate the tone and structure from blood vessels, which can increase the growth of VSMCs, cell migration, inflammation and deposition of cellular matrix proteins, resulting in a vascular remodeling process, including endothelial dysfunction and thickening of the vascular wall [128].

The $O_2^{-\bullet}$ has a very reactive unpaired electron, which causes high instability and very short life time of the molecule. Superoxide anion is generated through the reduction of molecular oxygen by different enzymes, such as NADPH oxidase, xanthine oxidase, COX, NOS, cytochrome P450 monooxygenase, mitochondrial respiratory chain enzymes, in all cell types, including VSMCs and endothelial cells [129–131]. Additionally, the three isoforms of NOS are capable of producing $O_2^{-\bullet}$ by a process called uncoupling [132,133], which consists of electrons transfer to O_2, instead of L-arginine.

$O_2^{-\bullet}$ reacts rapidly with NO and consequently reducing its half-life and starts the production of $ONOO^-$. $ONOO^-$ effects negatively producing a reduction in basal vasodilator tone, unbalancing the blood pressure control, increasing oxidative stress and producing endothelial damage [134]. Moreover, $O_2^{-\bullet}$ exerts vasomotor actions, since vasodilator and vasoconstrictor effects have been reported [135,136]. In small doses it can stimulate COX

causing the release of PGI_2, resulting in a vasodilator effect. On the other hand, at higher concentrations PGI_2 formation is inhibited, negatively affecting the balance in the vascular tone by stimulating the formation of $PGF_{2\alpha}$ and TXA_2.

$O_2^{-\bullet}$ can be reduced to H_2O_2, either spontaneously or enzymatically through dismutation by superoxide dismutase (SOD). In presence of the enzyme catalase or glutathione peroxidase, H_2O_2, is disrupted in water and oxygen. H_2O_2 is a stable molecule which can perform its effects locally near its production site, or diffuse through the cell membrane, since it is fat soluble can exert its effect on neighboring cells. This molecule modulates different aspects of endothelial cell function, such as growth and proliferation, apoptosis, cytoskeletal reorganization, inflammatory responses, vascular remodeling and regulation of endothelium-dependent vascular tone. H_2O_2 may possess dilating and constricting properties depending on tissue, experimental conditions or concentration [134]. Indeed, $O_2^{-\bullet}$ and H_2O_2 stimulate contraction after mobilizing the stored $[Ca^{2+}]_i$, and activating the Na^+/H^+ exchanger [77]. However, on precontracted vessels, $ONOO^-$ and H_2O_2 are able to induce vasodilation [137].

Neurotransmitters

The neurotransmitters released by the nervous endings from perivascular innervation exert action on the different cell types of the vascular wall through the union with its postsynaptic receptors [39,40]. The chemical nature and/or function of the neurotransmitters will depend on the animal species and the degree of innervation of the artery, being especially relevant in small caliber vessels, as is the case of mesenteric artery (**Table 1**).

Table 1. Vascular nerve regulation. Effect of different neurotransmitters released from their corresponding nerve endings.

Innervation	Neurotransmitter	Receptor	Location	Effect
Adrenergic	NA	α-adrenergic	Endothelium and VSMCs	vasoconstriction
		β-adrenergic		vasodilation
Cholinergic	ACh	Muscarinic M_1-M_5	Endothelium and VSMCs	Vasoconstriction and vasodilation
Nitrergic	NO	Difuses from neurons to vascular muscle cells		vasodilation
Serotoninergic	Serotonin	$5HT_1$-$5HT_7$	Endothelium and VSMCs	Vasoconstriction
peptidergic	VIP NPY CGRP P Substance	VPAC Y_1-Y_5 RAMP/CRLR NK1	Endothelium and VSMCs	Vaasoconstriction and vasodilation
Purinergic	ATP	P1-P2	Endothelium and VSMCs	Vasodilation

Sex hormones and regulation of vascular tone

Steroid hormones have been proven to participate in regulation of a variety of biological processes in the organism including modulation of blood pressure. In this regard, clinical and epidemiological studies have shown a higher prevalence of CVD in men than in age matched women, especially in pre-menopause [138]. However, after menopause the incidence of CVD increases, therefore, cardioprotective effects have been traditionally attributed to estrogens, while deleterious effects have been associated to androgens. However, more recent studies suggest that testosterone and other androgens have protective effects on cardiovascular function [29,139,140]. In this regard, most epidemiological studies have shown a high prevalence of low testosterone levels in men with coronary heart disease, and that this association exists regardless of the age of the patient. Also, low testosterone levels lead to insulin resistance, hypertension, dyslipidemia, and visceral obesity. These findings suggest that androgen deficiency in males contributesorchidectimez to the development and worsening of CVD [141,142]. Additionally, testosterone deficiency negatively affects the thickness of the carotid mid layer [143], increasing the median/lumen ratio. Consistent with this finding, the presence of sex hormones has also been seen to prevent internal remodeling in mesenteric arteries [144,145]. Inflammatory factors are also negatively affected due the low testosterone levels by influencing mediators of the inflammatory response, which include a C-reactive protein high sensitivity, and increased interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) levels [146]. However, the exact mechanism by which testosterone deficiency results in the worsening of CVD remains under research.

Male sex hormones are able to directly affect cardiovascular function through genomic and non-genomic actions on androgen receptors located on cell membranes, the cytosol and nucleus from endothelial cells, vascular smooth muscle, fibroblasts, myocytes and platelets [147,148].

In vascular tissues testosterone acts predominantly in endothelial cells and VSMCs. Indeed, a variety of studies have demonstrated that the key mechanism underlying the vasorelaxing action of testosterone is associated with the modulation of VSMCs membrane ion channels for Ca^{2+} and K^{+}, including inactivation of voltage operated Ca^{2+} channels (VOCC) [139,149] and an activation of K^{+} channels [150]. Both actions lead to vasorelaxation by respectively diminishing $[Ca^{2+}]_i$ and increasing hyperpolarization. In addition to the direct effects in testosterone on VSMCs, androgens possess the ability to alter the release and function of different endothelial factors that control vascular tone, including NO, ROS and prostanoids; since in arteries from orchidectomized rats decrease in NO release and/or

bioavailability, increase in oxidative stress and synthesis of vasoconstricting prostanoids has been reported [41,145,151–154].

Regarding testosterone involvement on prostanoids it has been demonstrated that sex hormones influence TXA_2 release and function. Orchidectomy increases TXA_2 by increasing COX-2 expression [154,155]. In addition, the vasoconstrictor response to TXA_2 and the TP receptors density is increased in vascular smooth muscle cells [156,157]. However, the effect of orchidectomy on TXA_2-induced contractile response depends on the vascular bed, since orchidectomized rats did not modify the response to the TXA_2 mimetic U-46619 in mesenteric arteries, while it increased this response in aorta [158].

It is known that sex hormones influence the vascular adrenergic function [159,160] although the mechanism by which this modulation occurs is still not fully understood. It has been shown a positive correlation between androgens and sympathetic activity have been described in men [161], and greater vascular resistance has been recorded in men compared to women, the difference being attributed to alpha-adrenergic activity [162]. *Ex vivo* studies have demonstrated in rat mesenteric arteries, that orchidectomy decreases the contraction induced by exogenous NA, altough the vasoconstrictor response to EFS is not modified [137]. Together all these data suggest that modulation of the adrenergic activity is one of the mechanisms by which androgens can alter peripheral vascular resistances. Concerning nitrergic innervation, in mesenteric arteries from orchidectomized rats, have shown a decreased nNOS expression, although the release of neuronal NO is mantained [137], because nNOS activity is increased through PKC activation [163]. However, NO metabolism is higher in segments from orchidectomized male rats due to the increased generation of superoxide anion (O_2^-) and peroxynitrite formation [137]. The alterations in the release and function of the different factors above explained, toghether with the activation of cell signaling pathways that lead to cell proliferation [145], may account for the altered vascular structure and functions as we observed in five-month post orchidectomized rats. In turn, this modiffications may be responsible for the development of hypertension in two-year old orchidectomized rats.

Non-pharmacological treatment of cardiovascular disseases

The prevention and treatment of cardiovascular diseases is still under research and constant improvement. Nowadays alternative pharmacotherapy such as vitamins, herbal remedies, and other dietary supplements (e.g. vitamin E, vitamin C, β-carotene, fish oils, garlic, soy, coenzyme Q10, and L-arginine) are utilized simultaneously or in replacement for traditional medical and interventional treatment. Most of the results of clinical studies on

alternative therapies used in cardiovascular disorders remain controversial, and risk-versus-benefit ratios are not well defined. However, these considerations have not deterred patients from trying these agents.

The evidence regarding fish oils- ω-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexenoic acid (DHA), on cardiovascular health is very wide. Multiple investigations, including *in vitro* studies, animal experiments, observational studies, and clinical trials, support these claims [164–166].

The unique three-dimensional structure and configuration of PUFAs ω-3 contributes to its influence on various metabolic pathways, which determine its physiological effects (**Figure 6**). PUFAs ω-3 have been shown to be involved in modifying membrane structure and function, tissue metabolism and gene regulation**.**

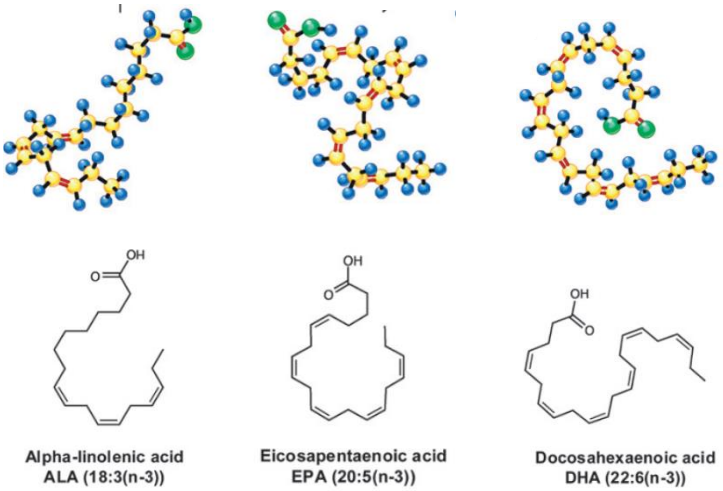

Figure 6. Major ω-PUFA. Major ω-PUFA include ALA, EPA, and DHA.The long hydrocarbon backbones, multiple double bonds, and location of the first double bond in the (n-3) position result in complex and unique 3-dimensional configurations that contribute to the singular biologic properties of these fatty acids. Reproduced from Mozzafarian *et al*. [167].

Generally, in humans and mammals, the biosynthesis of EPA and DHA from α-linolenic acid (ALA) is a process of low metabolic efficiency in the adult. From a nutritional point of view linoleic acid (LA) and ALA are commonly regarded as "essential" fatty acids, since they are not synthesized in the human body and are mainly obtained from external sources, like plants, for ALA, or marine foods for EPA and DHA. Its biosynthesis is a long process; ALA penetrates cells through cytoplasmic-specific fatty acid carriers and by acyl-CoA synthase is converted to thioesters, which undergo reactions of β-oxidation, elongation and desaturation, for the formation of EPA (20 carbons) and DHA (22 carbons), the conversion of ALA to EPA being less than 5% and in ALA to DHA being less than 1% [168]. This conversion is mainly performed in the liver, although it occurs to a lesser extent in the glial cells, testes, cardiomyocytes and

mammary gland [169]. The precursor of PUFA ω-6 is the LA, whose long chain fatty acid derivative is arachidonic acid (20 carbons), precursor of eicosanoids, such as prostanoids, which as mentioned above have pro-inflammatory and pro-thrombotic properties. ALA and LA compete in the same enzymatic pathway that leads to the generation of their long-chain fatty acids. Therefore, PUFAs concentration on tissues depend to a great extent on the amount of their respective precursors. Ingestion of fish or fish oil provides PUFA ω-3 directly, thus avoiding competition for enzymes in the conversion pathway [170].

Ω-3 PUFAs are responsible for the reduction of atherosclerotic lesions, a decrease in the frequency of cardiac arrest and a reduction in overall mortality in patients at risk for cardiovascular disease [155]. The beneficial effects on cardiovascular health attributed to PUFAs ω-3 are the result of their effects on the positive modification of the lipid profile, reduction of blood pressure, reduction of platelet aggregation and reduction of the incidence of arrhythmias, in addition to their anti-inflammatory and antioxidant effects. The American Heart Association recommends that patients with ischemic heart disease consume approximately 1 g/day of EPA + DHA and, in the absence of this disease, fish oil should be consumed at least 2 times a week. In the recommendations of the *European Society of Cardiology* for the treatment of acute myocardial infarction, supplementation with 1 g of ω-3 fatty acids was classified as a class I recommendation. Daily intake of ω-3 fatty acids recommended by the *Spanish Society of Community Nutrition* is 2.2 g (2 g ALA + 0.2 DHA).

As described above, diets enriched with fish oils with high content of DHA and EPA have multiple protective effects against CVD. On the other hand, it has been shown that vascular dysfunction is the first step towards CVD development and the relevance of sex hormones in maintenance of vascular function and structure in men has been established. Based on these data, it was hypothesized that a DHA-supplemented diet could prevent or ameliorate the vascular changes induced when an impaired gonadal function exists.

Taking into account the higher incidence of cardiovascular pathologies related to low testosterone levels such as hypogonadism, in prostate carcinomas whose treatment is based on the blockage of androgenic function, or the physiological process of aging itself coupled with an increased life expectancy with a consequent rise in aged population it is interesting to analyze the possible altered mechanisms involved in the regulation of vascular tone in absence of sex hormones in order to propose appropriate therapeutic treatments. Additionally, this study is of pathophysiological relevance since the results may provide evidence of the mechanisms involved in the protection of vascular function of the aorta (an elastic vessel) and the mesenteric artery (a resistant vessel), whose alterations lead to different cardiovascular diseases.

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Objectives

Objectives

The aim of this study was to analyze the effects of a DHA-supplemented diet in orchidectomized rats on different important parameters that contribute to the maintenance of vascular function, such as (1) lipid profile, (2) COPs, (3) redox status, (4) and inflammatory factors. In addition, responses to specific stimuli in isolated conductance and resistance vascular beds were also studied.

The first publication analyzed whether DHA supplemented diet affects the blood pressure, the lipid profile and redox status of serum samples from control and orchidectomized rats. The production of NO, prostanoids and the redox status (superoxide anion, hydrogen peroxide and the antioxidant capacity) were determined in aortic wall. Additionally, the endothelium dependent vasodilator response and the contribution of NO, prostanoids and hyperpolarizing factors on it were also investigated.

Since mesenteric vascular bed importantly regulates the systemic blood pressure, the second publication was aimed to analyze the influence of DHA in the prevention of functional alterations in the vascular function by monitoring more specifically the basal production of NO, prostanoids and ROS (superoxide anion). Regarding experiments on vascular function, the endothelium dependent vasodilator response, as well as the involvement of prostanoids and NO in that response were studied. Also, the vasoconstrictor responses induced by electrical field stimulation, and the vasomotor effect of the neurotransmitters NA and NO were analyzed.

Finally, the effect of the DHA supplemented diet on the formation of oxysterols in vascular wall of aorta and mesenteric arteries was collected in the third publication. The content of cholesterol oxidation products in vascular tissues was discussed in relation to the factors involved in aortic and mesenteric vascular regulation.

Publication 1:

Effect of dietary docosahexaenoic acid supplementation on the participation of vasodilator factors in aorta from orchidectomized rats

PLOS | ONE

RESEARCH ARTICLE

Effect of Dietary Docosahexaenoic Acid Supplementation on the Participation of Vasodilator Factors in Aorta from Orchidectomized Rats

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Abstract

Benefits of n-3 polyunsaturated fatty acids (PUFAs) against cardiovascular diseases have been reported. Vascular tone regulation is largely mediated by endothelial factors whose release is modulated by sex hormones. Since the incidence of cardiovascular pathologies has been correlated with decreased levels of sex hormones, the aim of this study was to analyze whether a diet supplemented with the specific PUFA docosahexaenoic acid (DHA) could prevent vascular changes induced by an impaired gonadal function. For this purpose, control and orchidectomized rats were fed with a standard diet supplemented with 5% (w/w) sunflower oil or with 3% (w/w) sunflower oil plus 2% (w/w) DHA. The lipid profile, the blood pressure, the production of prostanoids and nitric oxide (NO), and the redox status of biological samples from control and orchidectomized rats, fed control or DHA-supplemented diet, were analyzed. The vasodilator response and the contribution of NO, prostanoids and hyperpolarizing mechanisms were also studied. The results showed that orchidectomy negatively affected the lipid profile, increased the production of prostanoids and reactive oxygen species (ROS), and decreased NO production and the antioxidant capacity, as well as the participation of hyperpolarizing mechanisms in the vasodilator responses. The DHA supplemented diet of the orchidectomized rats decreased the release of prostanoids and ROS, while increasing NO production and the antioxidant capacity, and it also improved the lipid profile. Additionally, it restored the participation of hyperpolarizing mechanisms by activating potassium. Since the modifications induced by the DHA-supplemented diet were observed in the orchidectomized, but not in the healthy group, DHA seems to exert cardioprotective effects in physiopathological situations in which vascular dysfunction exists.

Introduction

The endothelium plays a pivotal role in the regulation of vascular tone through the release of vasoactive factors such as nitric oxide (NO), prostanoids, reactive oxygen species (ROS) and hyperpolarizing factors [1]. It is well known that NO-induced vasodilation is mediated through the activation of soluble guanylate cyclase, by increasing levels of cyclic guanosin monophosphate (cGMP) and cGMP-dependent protein kinase activity in the vascular smooth muscle of the arterial wall [2,3]. However, vascular functionality of endothelial NO depends on its bioavailability as NO can be metabolized into different ROS.

Prostanoids are synthesized from arachidonic acid metabolism through the cyclooxygenase (COX) pathway. Apart from their involvement in platelet aggregation and inflammation, they are also important regulators of vascular tone in health and disease [1,4]. Hyperpolarizing factors and mechanisms also participate in the regulation of vessel tone by activating potassium channels, which are responsible for the control of membrane potential [2,5]. Calcium-dependent (K_{Ca}) and ATP-dependent (K_{ATP}) potassium channels are widely expressed in vascular tissue [6,7]. The release of an endothelium-derived hyperpolarizing factor (EDHF) has been proposed and while the nature of EDHF remains to be defined, studies have suggested that hyperpolarization may result from endothelial release of different substances such as NO [8], superoxide anion [9] and other ROS [10].

Altered production and/or vascular effects of those factors could induce vascular dysfunction, leading to the development of different cardiovascular disorders such as atherosclerosis, hypertension and ischemia [1,11]. Over the years, different pharmacological treatments have been developed to mitigate the effects of these alterations such as antihypertensives, β-blockers, statins and aspirin [12–14]. Recently, studies have focused on nutritional interventions, with particular interest being paid to n-3 polyunsaturated fatty acids (PUFAs). For example, eicosapentaenoic acid (20:5n-3) or docosahexaenoic acid (DHA; 22:6n-3), that are found in seafood and fish oil supplements, due to their beneficial effects on cardiovascular diseases [15]. Antithrombotic, anti-inflammatory, and vasoprotector effects of PUFAs on the cardiovascular system have been reported [16–18]. Additionally, hyperpolarizing effects of PUFAs, contributing to the vasorelaxation through the activation of potassium channels have also been observed [19]. Nevertheless, controversy exists as not all studies provide a clear and conclusive idea regarding the benefits of PUFAs against cardiovascular diseases [20].

On the other hand, it is well established that sex hormones regulate vascular function by altering the release and cell signaling pathways of endothelial factors that could lead to vascular dysfunction. In this regard, we have demonstrated that the loss of gonadal function modifies the production of vasoconstrictor prostanoids [21–24], superoxide anion [25–27] and NO [28]. Since the number of vascular pathologies matching with decreased levels of sex hormones is increasing [29,30], it would be of interest investigate whether a DHA-supplemented diet could prevent vascular changes induced when an impaired gonadal function exists (i.e.: aging, hypogonadism, and pharmacological treatment of prostate cancer). Therefore, the objective of this work is to analyze how a DHA-supplemented diet affects the lipid profile, the blood pressure, the production of prostanoids and NO, and the redox status of biological samples from control and orchidectomized rats. The vasodilator response and the contribution of NO, prostanoids and hyperpolarizing factors were also analyzed.

Materials and Methods

Animals, diets and experimental groups

The protocol was approved by the Animal Ethics Committee of the Universidad Autónoma de Madrid (Ref. CEI-37-829) and procedures were performed according to the European Union directives 63/2010UE and Spanish regulation RD 53/2013. Male Sprague-Dawley rats (4 months old) were housed in the Animal Facility of the Universidad Autónoma de Madrid (Registration number ES-20079-0000097) under 12 hour light/dark cycles and standard feeding with fodder and water ad libitum. After 1 week of adaptation animals were fed a maintenance diet for rodents (Global Diet 2014, Harlan Laboratories Inc. Indianapolis, Indiana, USA) supplemented with fat (5%). The controls-diet groups were supplemented with sunflower oil (5%) and the DHA groups with 4.5% Marinol C-38 (lipid Nutrition) and adjusted to 5% with sunflower oil. Nutrient content and energy distribution of each diet is summarized in Table 1. After 2 weeks on the control- or DHA-diet, animals were divided into two subgroups: control (C) and orchidectomized (ORX) male rats. Male sex hormone deprivation was induced by orchidectomy at 18 weeks of age under anesthesia by isofluorane inhalation. Rats were treated with 0.30 mg/kg meloxicam SC (Metacam 5 mg/mL; Boehringer-Ingelhelm) immediately after surgery and with 50 mg/kg ibuprofen, via oral administration for 4 days. Animals were maintained on experimental diets for six more weeks. At the end of the treatment, rats were sacrificed (6 months old) by CO_2 inhalation and decapitation. The observation of seminal vesicles atrophy confirmed successful surgery. The aorta was carefully dissected out, cleaned of connective tissue and placed in Krebs-Henseleit solution (KHS) (containing, in mM: NaCl 115, $CaCl_2$ 2.5, KCl 4.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.1, Na_2 EDTA 0.03) at 4°C. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the USA National Institutes of Health (NIH publication No. 85.23 revised 1985). This study was approved by the Ethical Committee of the Universidad Autónoma de Madrid.

Table 1. Nutrient and energy content of experimental diets.

	Control Diet	DHA Diet
Carbohydrates (g/100g)	59.84	59.84
Protein (g/100g)	14.39	14.39
Total Fat (g/100g)	9.19	9.19
DHA + EPA		2.01
Energy (kcal/100g)	271.78	271.78

Blood pressure measurement

Systolic blood pressure was indirectly measured in awake animals by the tail-cuff method [23,24] before and after the treatment using a Letica Digital Pressure Meter LE5000 (Barcelona, Spain).

Blood biochemical analysis

Triglycerides, total cholesterol, LDL cholesterol, HDL cholesterol, glucose and creatinine in plasma were determined using an automated analyzer (Beckman Coulter-Former Olympus Diagnostics AU 5420, Nyon, Switzerland).

Release of prostanoids

After a stabilization period in KHS at 37°C for 30 minutes (pH 7.4), aortic rings from each group of rats was followed by 2 wash periods of 10 min using 0.2 mL of KHS. Once fresh KHS was replaced, arteries were exposed to 1 µM noradrenaline (NA) for 2 minutes and then to cumulative ACh concentrations (0.1 nM-10 µM) at 1-minute interval. Then, the medium was collected and stored at -80°C until used. Production of TXA_2, PGI_2, $PGF_{2\alpha}$ and PGE_2 induced by ACh, were monitored by measuring their stable metabolite TXB_2, 6-keto-$PGF_{1\alpha}$, 13, 14-dihydro-15-keto $PGF_{2\alpha}$ and PGE_2, respectively, using the respective enzyme immunoassay kit (Cayman Chemical). Results were expressed as pg prostanoid/mg tissue.

Production of nitric oxide

The fluorescent probe 4,5-diaminofluorescein was used to specifically evaluate NO production, as previously reported [25,26]. Briefly, aortic segments from each group of rats were cryoprotected with 30% w/v sucrose in PBS, frozen and stored at -80°C. After a washing period with PBS, the artery segments were opened to uncover the artery lumen to allow a better penetrance of the probe. Then the segments were immersed in N-(2-hydroxyethyl) piperazine-N0 2-ethane-sulfonic acid (HEPES) buffer (in mM: NaCl 119, HEPES 20, $CaCl_2$ 1.2, KCl 4.6, $KH_2PO_4$0.4, $MgSO_4$ 1, $NaHCO_3$ 5, glucose 5.5, $Na_2H_2PO_4$ 0.15; pH 7.4) containing 4,5-diaminofluorescein (0.5 µM), and incubated in a light-protected, humidified chamber at 37°C for 45 min. Then, the segments were mounted on glad slides and imaged on a confocal microscope. Images were obtained with a LEICA (TCS ST2 DM IRE2) laser scanning confocal microscope (excitation 495 nm, emission 515 nm). Laser and image settings were unchanged for the acquisition of images from the three groups of rats. The photomicrographs show the

intensity and location of 4,5-diaminofluorescein, which reflects NO production, so that comparison of these groups could be made. To analyze fluorescence intensity the ImageJ Analysis Software (National Institutes of Health) was used. Although DAF-2 is widely used as detector for NO, it can also react with intermediate products of the oxidation of NO. Therefore, in previous studies, experiments were performed in the presence of the NO synthesis inhibitor L-NAME, in which the fluorescence was abolished [26,31]. The amount of NO released was expressed as arbitrary units.

The basal production of NO was also measured by using a nitrite colorimetric assay kit (Cayman Chemical). Briefly, after a stabilization period in KHS at 37°C for 30 minutes (pH 7.4), aortic rings from each group of rats was followed by 2 wash periods of 10 min using 0.2 mL of KHS. Then, the medium was collected and stored at -80°C until assay performed. The assay was carried out according to the manufacturer's protocol and the absorbance was measured at 540 nm. Data were expressed as relative levels to control rats fed control-diet (= 1).

Detection of superoxide anion

Hydroethidine, an oxidative fluorescent probe, was used to evaluate superoxide anion levels *in situ*, as previously described [25,27]. Aortic segments from the four groups were cryoprotected with 30% (w/v) sucrose in PBS, frozen and stored at -80°C. After a washing period with PBS, the artery segments were opened to uncover the artery lumen to allow a better penetrance of the probe. Then the segments were immersed in HEPES buffer containing hydroethidine (5 µM), and incubated in a light-protected, humidified chamber at 37°C for 30 min. Segments were mounted on glad slides and imaged on a confocal microscope. Images were obtained with a LEICA (TCS ST2 DM IRE2) laser scanning confocal microscope (excitation 488 nm, emission 610 nm). Laser and image settings were unchanged for the acquisition of images from the three groups of rats. The photomicrographs show the intensity and location of hydroethidine, which reflects superoxide production, so that comparison of these groups could be made. To analyze fluorescence intensity the ImageJ Analysis Software (National Institutes of Health) was used. In a previous study, we reported that the fluorescence emitted by HE came from superoxide production since the fluorescence diminished in vessels pretreated with tempol, a membrane- permeable of SOD [25]. The amount of superoxide formation was expressed as arbitrary units.

Detection of hydrogen peroxide

Hydrogen peroxide levels in serum samples and aortic rings were measured by using a fluorescence H_2O_2 assay kit (Cayman Chemical). Serum samples from each group of rats were collected upon the day of sacrifice and, frozen at -80°C and used directly to perform the assay. Frozen samples of aortic segments were homogenized at 4°C in RIPA buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% (w/v) deoxycholic acid, 1% (v/v) NP-40, 1% (v/v) SDS, 100 mM NaF, 1 mM Na_3VO_4, and a protease inhibitor cocktail (Calbiochem) supplemented with freshly prepared 1 mM phenylmethylsulfonyl fluoride. Samples were centrifuged at 16,000 *g* during 30 min at 4°C and the supernatant was collected and used to the assay, which was carried out according to the manufacturer's instructions. The fluorescence at 530 and 590 nm excitation and emission wavelengths, respectively, was registered in a 96-microplate reader (Multiskan Ascent, Labsystems). Some assays were performed in the presence of catalase, an H_2O_2 scavenger, to ensure the specificity of the method. Supernatants were also used to quantify protein concentration by the bicinchoninic acid assay using the BCA™ Protein Assay Kit (Pierce). Data were expressed as µmol/µg protein for serum samples and nmol/mg protein for aortic tissue.

Detection of oxygen radical scavenging capacity (ORAC)

The antioxidant activity in serum samples and in aortic vascular wall was analyzed by using the hydrophilic oxygen radical scavenging capacity (ORAC) assay, as previously reported [32]. The sample processing was identical to that explained in the preceding section. The assay was carried out according to the manufacturer's instructions and the fluorescence read at 485 and 528 nm excitation and emission wavelengths, respectively (Multiskan Ascent, Labsystems). Data were expressed as values relative to control rats fed control-diet.

Vascular reactivity

The method used for isometric tension recording has been previously described [21,27,33]. In summary, aortic segments were suspended in an organ bath containing 5 mL of KHS at 37°C, continuously bubbled with 95% O_2–5% CO_2 mixtures (pH 7.4). Two parallel stainless steel pins were introduced through the lumen of the vascular segment: one fixed to the bath wall and the other connected to a force transducer (Grass FTO3C; Grass Instruments Co., Quincy, MA, USA); this in turn was connected to a model 7D Grass polygraph. The segments were subjected to a tension of 1 g which was re-adjusted every 15 min during a 90

min equilibration period before drug administration. After this, the vessels were exposed to KCl (75 mM) to check the functional integrity. After a washout period the viability of vascular endothelium was tested by the ability of 10 µMACh to relax precontracted segments with 0.1 µMNA.

Concentration-response curves to ACh (0.1 nM-10 µM) and to the nitric oxide donor sodium nitroprusside (SNP, 0.1 nM-10 µM) were performed in NA (0.1 µM) precontracted aortic rings from of the four groups of rats. To analyze the participation of NO and COX-derivatives on the response induced by ACh, the NO synthase inhibitor L-NAME (0.1 mM) and the nonselective inhibitor of COX-1/2, indomethacin (Indo, 10 µM), were added to the bath 30 minutes before performing the curve. To analyze the participation of hyperpolarizing factors on the responses to SNP and ACh, arteries were precontracted with 30 mM KCl, to block membrane hyperpolarization. To analyze the function of the K_{Ca} and K_{ATP} channels, concentration-response curves to the K_{Ca} and K_{ATP} channel openers NS1619 (0.1 nM-10 µM) and pinacidil (0.1 nM-10 µM) respectively, were also performed in NA (0.1 µM) precontracted aortic rings from each group of rats.

Reagents

Drugs used were: DAF-2, HE, L-NA hydrochloride, ACh chloride, L-NAME hydrochloride, indomethacin, potassium chloride, SNP, NS1619 and pinacidil (Sigma-Aldrich). Stock solutions (10 mM) of drugs were prepared in distilled water, except for NA which was dissolved in NaCl (0.9%)-ascorbic acid (0.01% w/v) solution, indomethacin in 1.5 mM NaHCO3 and NS- 1619 in dimethylsulfoxide. These solutions were kept at -20°C and appropriate dilutions were made in KHS on the day of the experiment.

Statistical analysis

Results are given as mean ± SEM (Standard Error of the Mean). The relaxation induced by ACh or SNP was expressed as a percentage of initial contraction elicited by NA. Statistical analysis was performed by comparing the curve obtained in the presence of the different substances with the control curve by means of two-way analysis of variance (ANOVA). For blood pressure, body weight, biochemical parameters, prostanoids, NO, superoxide, hydrogen peroxide production and ORAC, statistical analysis was done using Student's t-test for unpaired experiments. A p value of less than 0.05 was considered significant.

Results

Animal weight, systolic blood pressure and biochemical parameters

Before DHA administration, body weight and blood pressure were evaluated in the four groups of animals, showing no statistically significant differences among the groups (Table 2). After six weeks under the experimental diet, no significant changes in blood pressure were found. All animals had increased in body weight to a similar extent (Table 2). As shown in Table 3, orchidectomy induced a significant increase in total cholesterol concentrations and LDL cholesterol, although HDL cholesterol was not statistically modified. Triglyceride concentrations were also increased by the orchidectomy. The DHA-supplemented diet to the orchidectomized rats decreased the total cholesterol, LDL cholesterol and triglyceride levels. However, the control rats fed the DHA-diet decreased only the total cholesterol concentration. No differences were observed in serum concentration of glucose among groups.

Table 2. Systolic blood pressure (mmHg) and body weight (g) in control (C) and orchidectomized (ORX) rats fed with a control or DHA-supplemented diet.

	Blood pressure (mm Hg)		**Body weight (g)**	
Animal group	Before diet	After diet	Before diet	After diet
C Control (9)	149.6 ± 3	148.2 ± 3	390.9 ± 10	502.4 ± 20
C DHA (7)	145.4 ± 4	142.4 ± 6	394.9 ± 7	484.9 ± 12
ORX Control (8)	150.2 ± 2	146.4 ± 5	385.4 ± 8	477.5 ± 18
ORX DHA (11)	146.6 ± 3	143.5 ± 1	411.6 ± 10	506.5 ± 9

Values are means ± SEMs. The number of animals used is indicated in parenthesis.

Table 3. Serum lipid profile and glucose concentration in control (C) and orchidectomized (ORX) rats fed with a control or DHA-supplemented diet.

Serum biomarkers (mg/dL)	**C Control**	**C DHA**	**ORX Control**	**ORX DHA**
Cholesterol	121.4 ± 5.55	89.0 ± 14.57[a]	153.6 ± 6.49[a]	91.25 ± 6.42[b]
HDL Cholesterol	68.25 ± 4.29	59.28 ± 6.63	78 ± 4.68	70.2 ± 5.85
LDL Cholesterol	33.15 ± 3.12	26.13 ± 5.07	49.92 ± 3.9[a]	24.18 ± 2.73[b]
Triglycerides	99.20 ± 5.74	80.33 ± 18.89	141.8 ± 9.63[a]	83.60 ± 6.8[b]
Glucose	121.2 ± 12.85	150.3 ± 8.5	127.0 ± 8.4	113.0 ± 20.81

Values are mean ± SEMs. Number of animals per group, n = 5.

[a] Indicates difference with C Control group values.

[b] Indicates differences with the ORX group values.

Release of prostanoids

Orchidectomy increased the ACh-stimulated aortic production of TXA_2, PGI_2, $PGF_{2\alpha}$ and PGE_2 and PGE_2 (Fig 1). These values were restored to similar levels found in aorta from control animals after dietary DHA supplementation. In contrast, the DHA-diet in control rats decreased PGE_2 release and did not significantly modify the release of TXA_2, PGI_2, $PGF_{2\alpha}$ (Fig 1).

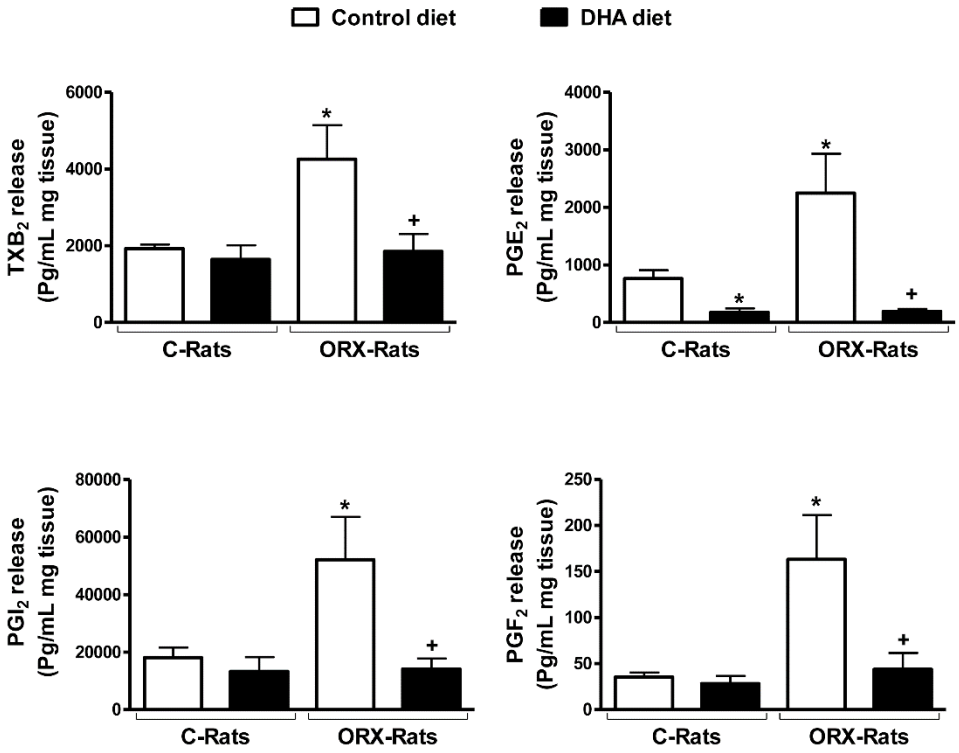

Fig 1. Effect of orchidectomy and a DHA-supplemented diet on basal release of prostanoids from rat aorta. Release of thromboxane B_2 (TXB_2), prostaglandin E_2 (PGE_2), PGI_2 and $PGF_{2\alpha}$ in aortas from control (C) and orchidectomized (ORX) rats fed with a control or with a DHA-supplemented diet. Values are means ± SEMs. Number of animals, n = 5. *P < 0.01 compared with C rats fed Control diet; +P < 0.01 compared with ORX rats fed DHA-diet.

Production of nitric oxide

After incubation with 4,5-diaminofluorescein, the fluorescence emitted was decreased in arteries from orchidectomized rats respect to arteries from control rats, and it was recovered after DHA-supplemented diet to orchidectomized rats; however, the DHA-diet did not modify the fluorescence emitted in arteries from control rats (Fig 2A). Relative basal measurement of nitrite levels shown similar results (arteries from control rats-control diet: 1; orchidectomized rats-control diet: 0.14 ± 0.06, $p < 0.05$ vs control diet; control rats-DHA diet:

0.98 ± 0.3, $p > 0.05$ vs control diet; orchidectomized rats-DHA diet: 0.77 ± 0.15, $p < 0.005$ vs control diet; n = 5).

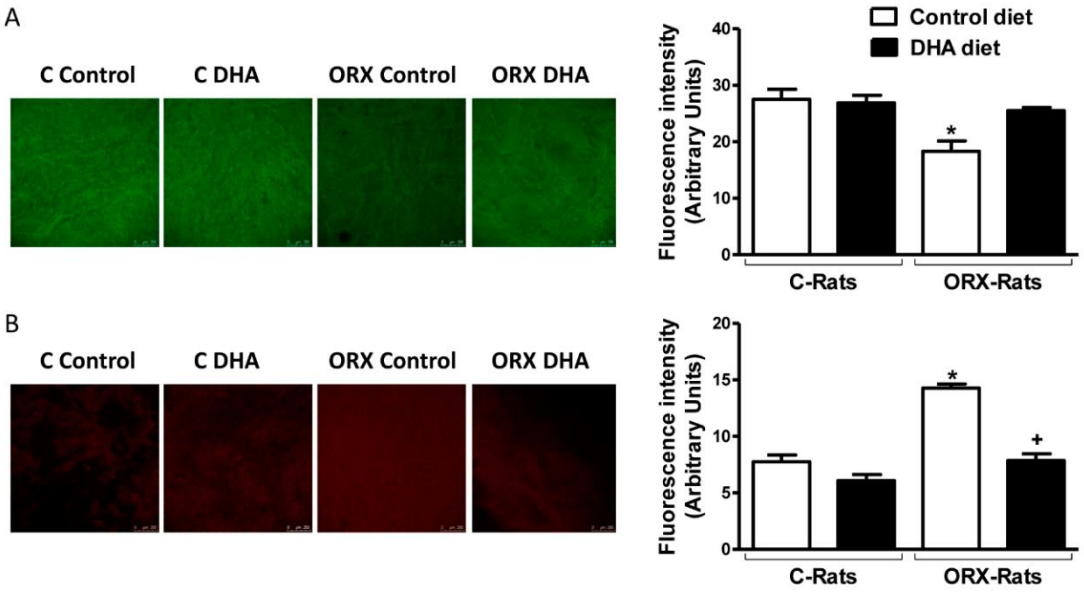

Fig 2. Effect of orchidectomy and the DHA-supplemented diet on the production of endothelial NO and superoxide anion from rat aorta. Confocal micrographs showing *in situ* detection of NO (A) or superoxide anion (B) in aortic segments from control (C) and orchidectomized (ORX) rats fed with a control diet or with a DHA-supplemented diet (DHA). The sections shown are typical preparations from five rats. Quantitative analysis of fluorescence is also shown. Values are means ± SEMs. Number of animals, n = 5. *$P < 0.01$ compared with control animals; $^{+}P < 0.01$ compared with ORX rats control diet

Detection of superoxide anion

After incubation with hydroethidine, the arteries from orchidectomized rats showed a markedly higher level of fluorescence than the arteries from control rats. This increase was reduced in the vessels from orchidectomized rats fed with the DHA-supplemented diet. In contrast, the DHA diet did not affect the production of superoxide anion in control animals (Fig 2B).

Detection of hydrogen peroxide

The levels of hydrogen peroxide, in serum samples and aortic wall, were increased in orchidectomized rats, which were returned to control level when orchidectomized rats fed with the DHA-supplemented diet. The DHA-diet to control animals did not modify hydrogen peroxide levels (Fig 3).

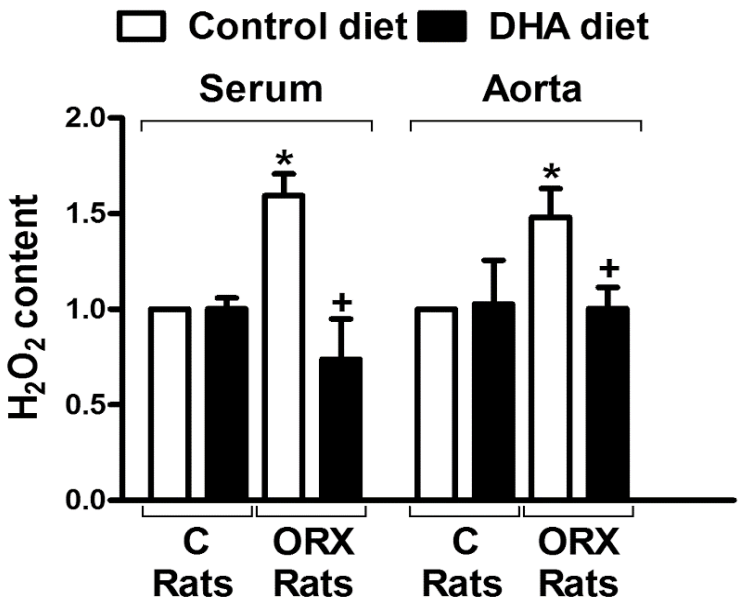

Fig 3. Effect of orchidectomy and the DHA-supplemented diet in the H_2O_2 content in rat serum and aorta. Relative levels of H_2O_2 in serum and aortic rings from control (C) and orchidectomized (ORX) rats fed with a control or with a DHA-supplemented diet. Values are means ± SEMs. Number of animals, n = 4. *$P < 0.01$ compared with control animals; $^{+}P < 0.01$ compared with ORX rats control diet.

Antioxidant activity

The antioxidant activity in both serum and aortic wall was decreased by the orchidectomy. DHA-diet to orchidectomized rats restored the activity up to levels similar shown in the control rats. However, the DHA-diet failed to modify the antioxidant activity in the serum from control animals, and surprisingly decreased the activity level in the aortic vascular wall (Fig 4).

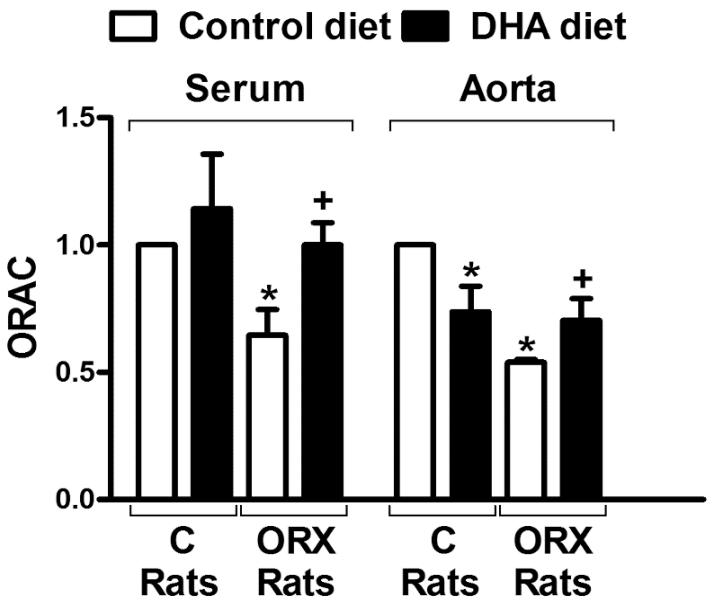

Fig 4. Effect of orchidectomy and the DHA-supplemented diet in the antioxidant activity from rat serum and aorta. Relative levels of H_2O_2 in serum and aortic rings from control (C) and orchidectomized (ORX) rats fed with a control or with a DHA-supplemented diet. Values are means ± SEMs. Number of animals, n = 6. *P < 0.01 compared with control animals; ^{+}P < 0.01 compared with ORX rats control diet.

Vascular reactivity

The vasodilator response induced by ACh (0.1 nM-10 µM) in aortic segments precontracted with NA (0.1µM) was similar in the four groups of rats (Fig 5). The vasodilator response induced by the NO donor SNP was not modified in any group (Fig 5), indicating that the sensitivity of NO on smooth muscle was intact.

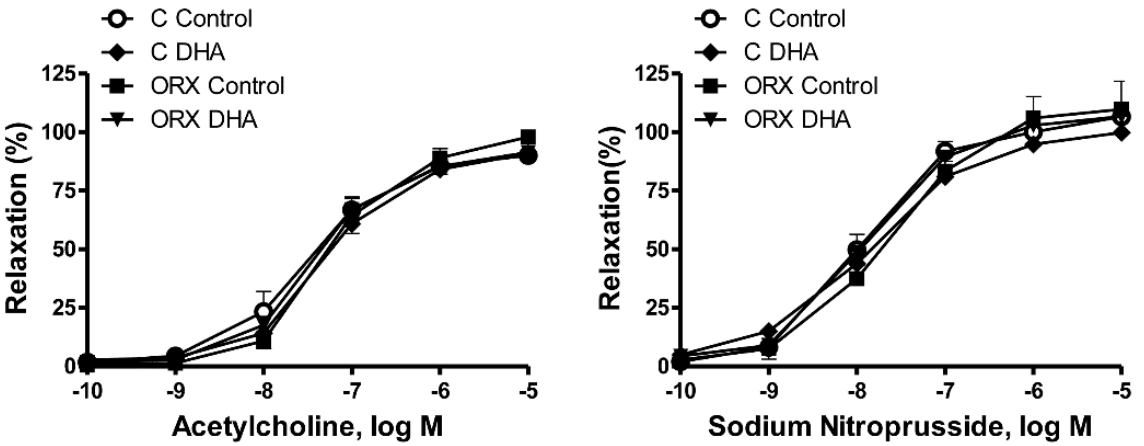

Fig 5. Effect of orchidectomy and a DHA-supplemented diet on the concentration-response curves to acetylcholine and sodium nitroprusside in rat aorta. Concentration-response curves to acetylcholine and sodium nitroprusside in aortic segments from control (C) and orchidectomized (ORX) rats fed with a control or a DHA-supplemented diet. Results (means ± SEMs) are expressed as percentage of inhibition of the contraction induced by 0.1 µM noradrenaline. Number of animals, n = 5–8.

To investigate the contribution of NO or prostanoids on the vasodilator response induced by ACh, the effect of the inhibitors of NO or prostanoids synthesis, L-NAME or indomethacin, was examined. In the presence of L-NAME the ACh-induced relaxation was inhibited in aortic segments from control or orchidectomized rats; the control-diet did not alter the inhibitory effect of L-NAME, while in segments from orchidectomized rats supplemented with DHA a small relaxation occurred (Fig 6). Incubation with indomethacin decreased the ACh-induced relaxation response in arteries from control rats fed control diet, while it did increase the ACh-induced response when the animals were fed DHA-diet. In aortas from orchidectomized rats fed control- or DHA-diet, indomethacin did not modify the Ach-induced response (Fig 6).

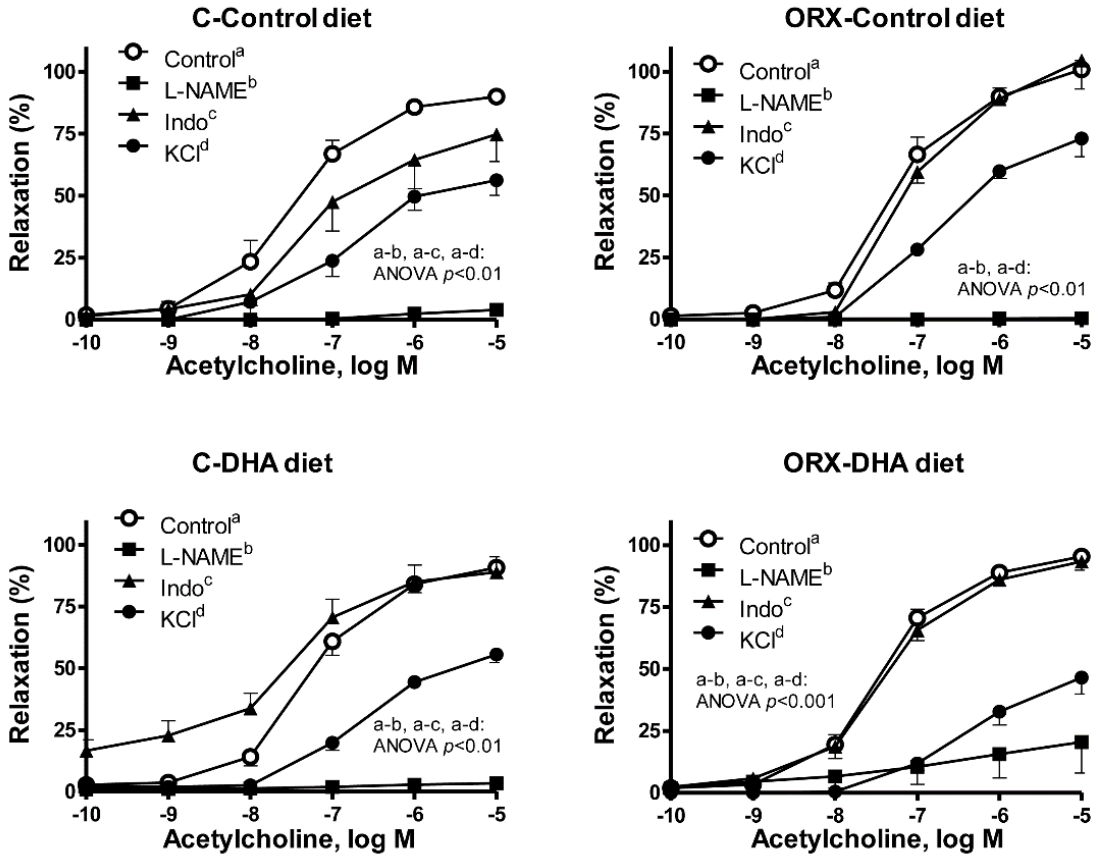

Fig 6. Orchidectomy and DHA-supplemented diet modulate the participation of different factors in the Ach-induced responses. Effect of L-NAME (0.1 mM) or indomethacin (Indo, 10 μM) on the concentration response curves to acetylcholine in the NA-precontracted aortic segment from control (C) and orchidectomized (ORX) rats fed with a control diet or with a DHA-supplemented diet (DHA diet). The effect of precontracting vessels with KCl (30 mM) is also represented. Results (means ± SEMs) are expressed as percentage of inhibition of the contraction induced by 0.1 μM NA or 30 mM KCl. Number of animals, n = 5–8.

The possible participation of hyperpolarizing mechanisms in the ACh-induced response was also analyzed by precontracting vessels with KCl (30 mM) that blocks the membrane hyperpolarization. Under this condition the ACh-induced response was decreased in arteries from the four groups of animals (Fig 6), but in a greater extent in aortas from orchidectomized animals fed with a DHA supplemented diet.

Since NO can also hyperpolarize the membrane of smooth muscle cell, the participation of hyperpolarizing mechanisms on the relaxation induced by SNP was also analyzed. In KCl-precontracted arteries the relaxation induced by SNP was reduced in the four groups of animals (Fig 7).

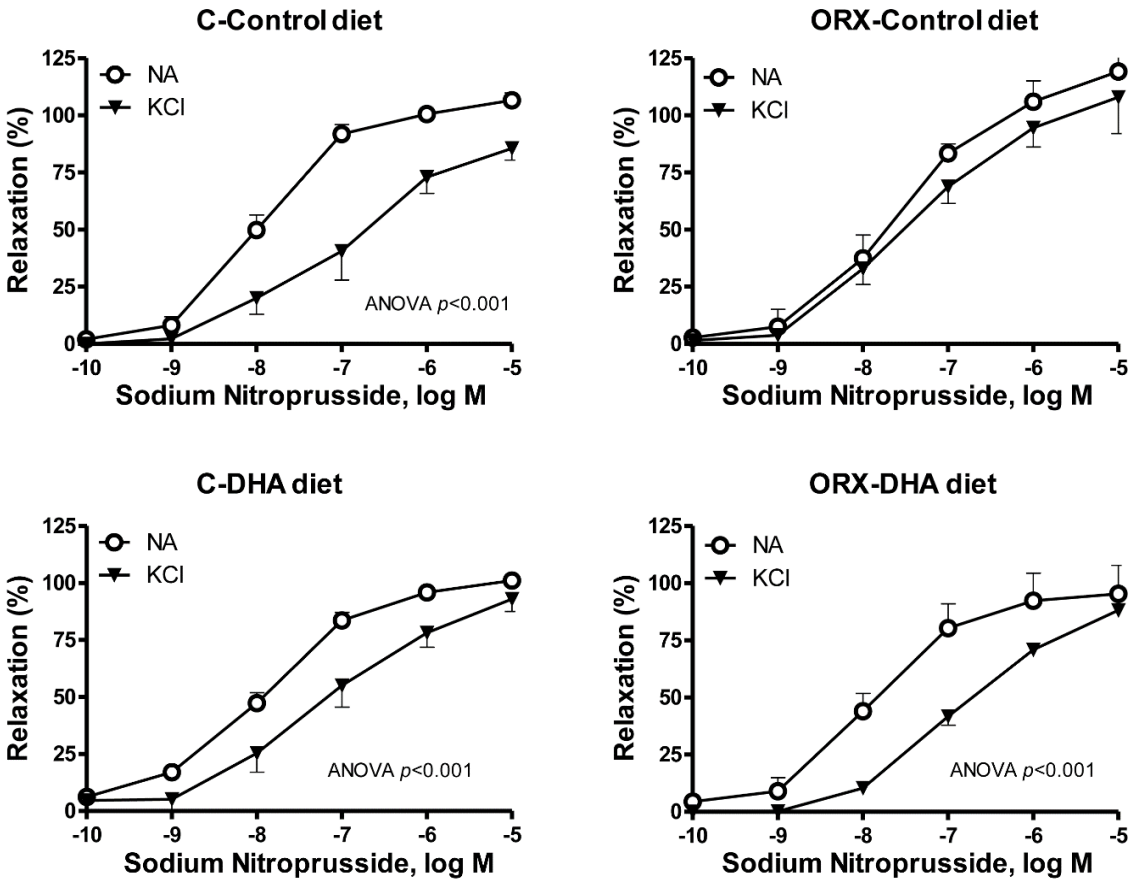

Fig 7. Orchidectomy and DHA-supplemented diet modulate the involvement of hyperpolarizing mechanisms in the sodium nitroprusside-induced response. Concentration-response curves to sodium nitroprusside in NA- or KCl-precontracted aortic segments from control (C) and orchidectomized (ORX) rats fed with a control diet or with a DHA-supplemented diet (DHA diet). Results (means ± SEMs) are expressed as percentage of inhibition of the contraction induced by 0.1 μM NA or 30 mM KCl. Number of animals, n = 4–7.

The SNP-induced relaxation was decreased in a greater extent in arteries from control rats than in those from orchidectomized rats. The DHA-diet did not significantly modify the response in arteries from control rats, while it increased the blockage in arteries from orchidectomized (Fig 7).

Since the activation of the K_{Ca} and K_{ATP} channels causes vasodilation by hyperpolarizing cell membrane, the effects of orchidectomy and DHA supplementation on the function of the K_{Ca} and K_{ATP} channels were analyzed. Concentration-response curves to the K_{Ca} and K_{ATP} channels openers NS1619 (0.1 nM-10 µM) and pinacidil (0.1 µM-10 µM), respectively, were analyzed in NA (0.1 µM) precontracted aortic rings from the four groups of rats (Fig 8). Orchidectomy partially decreased the vasodilator response induced by NS1619, which was recovered in the orchidectomized DHA group. Orchidectomy decreased the pinacidil-induced response, which was also recovered after DHA-supplemented diet. However, the DHA-diet failed to modify the NS1619- and pinacidil-induced responses in arteries from control rats (Fig 8).

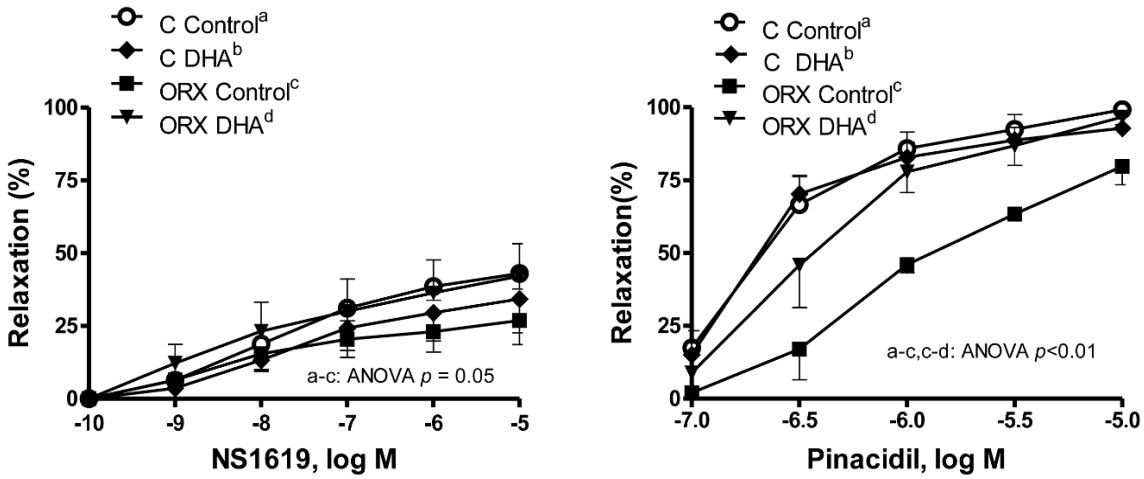

Fig 8. Effect of orchidectomy and the DHA-supplemented diet on the function of K_{Ca} and K_{ATP} channels. Concentration-response curves to the K_{Ca} and K_{ATP} channels openers NS1619 and pinacidil in NA-precontracted aortic segments from control (C) and orchidectomized (ORX) rats fed with a control diet or with a DHA-supplemented diet (DHA). Results (means ± SEMs) are expressed as percentage of inhibition of the contraction induced by 0.1 µM NA. Number of animals, n = 4–6.

Discussion

The present work provides information on the different impact of a DHA-supplemented diet to healthy or orchidectomized rats, analyzing the possible modifications in the lipid profile, in the release of different vascular factors and in the redox status that control vascular function. The interest of this particular experimental model is because the number of vascular pathologies matching with decreased levels of sex hormones (i.e.: aging, hypogonadism, and pharmacological treatment of prostate cancer) is increasing [29,30]. We have previously demonstrated that the release of prostanoids was increased 5 months post-orchidectomy in aorta and mesenteric arteries [21–23,25], most likely as a consequence of increased oxidative stress observed in these experimental conditions. In the present study we show that in aorta from 6 weeks post-orchidectomized rats the ACh-induced release of PGI_2, $PGF_{2\alpha}$ and PGE_2 were already increased, as observed for TXA_2 release in a previous study [28]. These results showed that the loss of gonadal function favored the onset of a pro-inflammatory environment. Dietary supplementation with DHA prevented the increase in the prostanoids release induced by orchidectomy, which agrees with the described anti-inflammatory properties of PUFAs [16,17,34]. However, in control animals, this property was shown for PGE_2, that was decreased, which is in line with most of the studies describing a reduction after PUFAs-diet in healthy subjects [35,36]. The DHA-diet did not modify the production of TXA_2, PGI_2 and $PGF_{2\alpha}$ in control rats, as previously reported for TXA_2 production [37].

Regarding the production of NO, quantified by measuring nitrite production and the fluorescence emitted by DAF-2, the current study shows that it was decreased in aortas from 6 weeks post-orchidectomized rats, according to previously published [28], while in aortas from orchidectomized rats fed a DHA-supplemented diet the NO production was restored. These results are in line with other studies describing increase in NO release by n-3 PUFA [38–40]. However, the DHA-diet did not significantly modify the release of NO in arteries from control rats, as reported by Omura and coworkers [41] who observed that although the EPA increased NO release, DHA had no effect. This result indicates that the effect on NO release may depend on the specific PUFA tested. But what is more important is that the effect of a specific PUFA, DHA in our study, on the prostanoids and NO release seems to depend on the baseline conditions of the experimental treated animals.

Oxidative stress is one of the major detrimental events in the induction of vascular dysfunction. Since we already reported that 5 months post-orchidectomy increased the production of superoxide anion [25,26,31], we now analyzed the effect of the DHA-

supplemented diet on the ROS production. The results showed that 6 weeks post-orchidectomy already increased the production of superoxide in the aortic wall, while in arteries from orchidectomized rats fed the DHA-supplemented diet the production of superoxide was diminished to a level similar of that found in arteries from control rats. However, the aorta from control rats fed DHA-diet did not modify the production of superoxide. Thus, superoxide production has been decreased by n-3 PUFAs in platelets from diabetic hypertensive patients [42]. DHA protects endothelial cells culture against oxidative stress through Nrf2 activation [43] and down-regulating Nox4 [44]. These results suggest that DHA interfere with the oxidative stress in pathophysiological situations in which there is a ROS overproduction. It is important to mention that although in previous studies we reported that the fluorescence emitted by hydroethidine came from superoxide-derived products [25,27], the specificity of this probe has been questioned because it could generate artefacts [45–47]. Therefore, it would be desirable to use other methods in future studies. Nevertheless, since superoxide can be metabolized to hydrogen peroxide, which was increased in mesenteric arteries from orchidectomized rats [25], and that exerts important vascular effects [25, 27] we also analyzed the presence of this compound in serum and aortic wall. The results shown that orchidectomy increased the hydrogen peroxide content in both serum and aortic samples, which were restored by the DHA-diet. These results are in agreement with the modification in the superoxide anion production commented above. Here again, control rats fed DHA-diet did not modify the hydrogen peroxide content respect to that observed with control diet. In view of these results, the oxygen radical scavenging capacity in serum and aortic wall was also studied. As expected, orchidectomy decreased the antioxidant activity, which was recovered in the orchidectomized rats fed DHA-diet. DHA-diet failed to modify the antioxidant activity in samples from control animals. The overall results on the redox status demonstrates the antioxidant ability of DHA [48,49] and provides evidence for a positive effect of DHA-supplemented diet to prevent vascular dysfunction caused by an imbalance between the formation and elimination of ROS.

In addition to changes in the production of NO, prostanoids and ROS induced by orchidectomy, the lipid profile is also under control of the androgens levels, and importantly affects the vascular function. In this regard, the orchidectomized group fed the control-diet showed an increase in total cholesterol, LDL-cholesterol and triglycerides, according to the effect of androgen deprivation on lipid profile [50]. However, the DHA-diet normalized the lipid levels in orchidectomized rats while in control animals diminished only the total cholesterol level, without modifying the rest of parameters. These results are in line with previous human [51] and animal [52] studies showing that PUFAs intake improves the altered

lipid profile. Importantly, despite the changes in the production of NO, prostanoids, ROS and in the lipid profile in the ORX and ORX-DHA group, the systolic blood pressure was not modified in any group. The lack of effect of a DHA-supplemented diet on blood pressure is in agreement with other studies where no effects were noted in the short term [53]. However, a decrease in blood pressure has also been reported in both spontaneously hypertensive [54,55] and aged [53] rats. Additionally, our results also suggest that the alterations in vascular function described below are independent of hemodynamic changes.

Regarding endothelium-dependent relaxation, it was shown that 6-weeks post-orchidectomy did not modify the ACh-induced relaxation. The DHA-supplemented diet to control or orchidectomized rats did not alter the ACh-induced relaxation, which is in agreement with other studies in aged [53], hypertensive [55] or diabetic [56] rats. However, other studies have revealed that the intake of n-3 PUFAs improves the NO-mediated endothelium-dependent vasodilation in patients with coronary artery disease [57]. These results suggest that the influence of DHA on the endothelium dependent vasorelaxation is controversial and appears to mainly depend on the experimental conditions and the animal species. It is noteworthy that while the release of prostanoids and NO are modified by the orchidectomy and restored by the DHA-diet, the endothelial-dependent vasodilator responses induced by ACh remain unchanged in vessels from the four groups of rats. However, the contribution of different factors such as NO, prostanoids or hyperpolarizing mechanisms cannot be ruled out. We observed that NOS inhibition with L-NAME blocked the ACh-induced response in aortas from control rats with control- or DHA-diet, indicating that the vasorelaxant response to ACh is mainly due to the NO, as described for large elastic arteries [58]. In aortas from orchidectomized rats, the ACh-induced relaxation was also abolished by L-NAME, which coincides with the diminished production of NO reported in aorta from these animals. However, in aorta from orchidectomized rats fed with DHA, L-NAME did not stop the ACh-induced response, as a very small relaxation can be observed. Considering that the sensitivity of the smoothmuscle to NO was not modified, these results indicate that although NO is the most important factor in the response to ACh, other mechanisms may be contributing to the relaxation in this group of vessels. Therefore, the participation of prostanoids in the ACh-induced response were analyzed. The results showed that the inhibition of COX-1 and COX-2 with indomethacin decreased the vasodilator action of Ach in control rats, indicating the involvement of vasodilator net effect of prostanoids in this response. However, in aorta from orchidectomized rats the ACh-induced response was not modified suggesting that the net vasodilator effect has been lost. This is supported by the increased production of vasoconstrictor prostanoids, which was discussed above. Furthermore, when prostanoid

synthesis is inhibited the formation of NO is modified [22], indicating that NO may also be involved.

Despite the decreased production of prostanoids, in arteries from orchidectomized rats fed the DHA-diet, indomethacin did not modify the ACh-induced response, which could be attributed to that DHA could reduce the sensitivity of the smooth muscle to prostanoids [59,60]. More surprising was the effect of DHA-diet in control rats, in which an increase in the Ach response in presence of indomethacin was observed, indicating the prevalence of a net vasoconstrictor effect of prostanoids. This result may be related to the decrease in PGE_2, and indicates that intake of DHA supplements not would have a beneficial effect on the vasodilator response in healthy subjects.

Since the endothelium is able to synthesize EDHF and/or substances that hyperpolarize the cell membrane [8–10], the participation of hyperpolarizing mechanisms in the ACh-induced response was analyzed. Our results show that after blocking hyperpolarization [61], the response to ACh was decreased in all groups of animals, but to a greater extent in aortas from orchidectomized rats with DHA-supplemented diet. This suggests a participation of hyperpolarizing mechanisms in the relaxation induced by ACh, in particular when orchidectomized animals are fed a DHA-supplemented diet. These results are in agreement with the hyperpolarizing mechanisms induced by DHA [17]. In view of these results, the hyperpolarizing effect of NO was also investigated. These results indicate that NO partially hyperpolarizes the cell membrane and that orchidectomy decreases the degree of SNP-induced hyperpolarization, which was restored by the DHA-diet. It is well known that K_{Ca} and K_{ATP} channels have an important role on vascular tone regulation [5,7] and that their function may be modulated by sex hormones [62–64] and PUFAs [17,65,66]. Taking into account that orchidectomy decreases the participation of hyperpolarizing mechanisms induced by ACh or SNP, the vasodilator response to the K_{Ca} and K_{ATP} channels openers NS1619 and pinacidil, respectively, were analyzed. We observed that orchidectomy decreased the NS1619- and pinacidil-induced responses, which is in line with the hyperpolarizing effects caused by the acute administration of testosterone [62–64]. In arteries from orchidectomized DHA-supplemented rats, the vasodilator response elicited by the potassium channels openers was restored up to similar levels to those found in arteries from control rats. Therefore, the effect of DHA supplementation is in agreement with other studies where increased activation of K_{Ca} [19,65,66] and K_{ATP} [67,68] channels have been reported. However, the DHA-diet did not increase the activation of K channels in control to rats, pointing out that the choice of experimental models is crucial to determine the actions of a DHA-supplemented diet.

The results showed in the present study are of pathophysiological relevance since they provided mechanisms involved in the regulation of elastic artery function. The maintenance for long periods of an increased oxidative stress and chronic inflammation increase the proteolytic activity of the extracellular matrix, which could affect the integrity of aortic wall and ultimately lead to vascular damage as observed in aortic aneurysms [69].

In summary, the results obtained in aortas from male rats showed that orchidectomy induced the following effects: i) increased the production of prostanoids and ROS; ii) decreased NO production and the antioxidant capacity; iii) negatively affected the lipid profile; and iv) decreased the participation of hyperpolarizing mechanisms in the vasodilator responses, in which K_{Ca} and K_{ATP} channels are involved. The DHA-supplemented diet of the orchidectomized rats exerted anti-inflammatory and antioxidant effects by decreasing the release of prostanoids and ROS, while increasing NO production and the antioxidant capacity, and it also improved the lipid profile. Additionally, it increased the participation of hyperpolarizing mechanisms by activating K_{Ca} and K_{ATP} channels.

Since the modifications induced by the DHA-supplemented diet were observed only in the orchidectomized but not in the healthy group, the overall results show that DHA exerts cardioprotective effects in physiopathological situations in which vascular dysfunction exists. To further explore the mechanisms of action of PUFAs in health and disease to explain these differences more studies will be required.

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Author Contributions

Conceived and designed the experiments: MF HSG CO. Performed the experiments: DMV RN LdC. Analyzed the data: DMV RN LdC MF. Contributed reagents/materials/analysis tools: MF CO HSG CL MT DM RB. Wrote the paper: MF.

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Docosahexaenoic acid supplemented diet influences the orchidectomy-induced vascular dysfunction in rat mesenteric arteries

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RESEARCH ARTICLE

Docosahexaenoic Acid Supplemented Diet Influences the Orchidectomy-Induced Vascular Dysfunction in Rat Mesenteric Arteries

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Abstract

Over the past few decades, the cardiovascular benefits of a high dietary intake of long-chain polyunsaturated fatty acids (PUFAs), like docosahexaenoic acid (DHA), have been extensively studied. However, many of the molecular mechanisms and effects exerted by PUFAs have yet to be well explained. The lack of sex hormones alters vascular tone, and we have described that a DHA-supplemented diet to orchidectomized rats improve vascular function of the aorta. Based on these data and since the mesenteric artery importantly controls the systemic vascular resistance, the objective of this study was to analyze the effect of a DHA-supplemented diet on the mesenteric vascular function from orchidectomized rats. For this purpose mesenteric artery segments obtained from control, orchidectomized or orchidectomized plus DHA-supplemented diet were utilized to analyze: (1) the release of prostanoids, (2) formation of NO and ROS, (3) the vasodilator response to acetylcholine (ACh), as well as the involvement of prostanoids and NO in this response, and (4) the vasoconstrictor response to electrical field stimulation (EFS), analyzing also the effect of exogenous noradrenaline (NA), and the NO donor, sodium nitroprusside (SNP). The results demonstrate beneficial effects of DHA on the vascular function in orchidectomized rats, which include a decrease in the prostanoids release and superoxide formation that were previously augmented by orchidectomy. Additionally, there was an increase in endothelial NO formation and the response to ACh, in which NO involvement and the participation of vasodilator prostanoids were increased. DHA also reversed the decrease in EFS-induced response caused by orchidectomy. All of these findings suggest beneficial effects of DHA on vascular function by reversing the neurogenic response and the endothelial dysfunction caused by orchidectomy.

Introduction

The involvement of endothelial, hormonal and neural factors in the regulation of vascular function is well established [1, 2], although the contribution of these factors depends on the type of the vessel. In response to different stimuli the endothelium can release different factors, such as nitric oxide (NO), prostanoids, reactive oxygen species (ROS), among others [1]. NO is a signaling molecule formed by the enzyme nitric oxide synthase (NOS) that plays a crucial role in vascular homeostasis regulating the vascular tone, and therefore also influences blood pressure. This molecule exerts vasodilation in smooth muscle cells by stimulating the protein kinase G (PKG) trough soluble guanylate cyclase (sGC) in the smooth muscle of the arterial wall [3]. Also, NO has anti-inflammatory, antithrombotic, antiproliferative, and antioxidant effects. A decrease in NO synthesis and/or bioavailability leads to the development of vascular dysfunction [4].

The endothelium is also a source of ROS generated through the activation of xanthine oxidase, cyclooxygenase, and cytochrome P-450 [5, 6]. Excessive production of ROS, causes vascular dysfunction by outstripping endogenous antioxidant defense mechanisms, and it has been implicated in the pathogenesis of many cardiovascular diseases, including hypercholesterolemia, atherosclerosis, hypertension, diabetes, and heart failure [7].

The vascular tone is also regulated by prostanoids originating from arachidonic acid metabolism through the cyclooxygenase (COX) pathway [8]. Prostanoids are involved in platelet aggregation and inflammation, playing an important role in the regulation of vascular tone in physiopathological conditions. Vasodilator prostanoids such as prostacyclin and prostaglandin E_2 (PGE_2) play a role in parallel with NO in the regulation of vascular tone and blood pressure. In comparison, TXA_2 is considered a potent vasoconstrictor and increased production of this factor is correlated with alterations in vascular functions [9].

Rat mesenteric artery possesses nitrergic and sympathetic innervations in which the release of NO and noradrenaline (NA) are involved in the neuronal regulation of vascular tone [10±12]. Upon release, NO induces vasodilator action as commented above. NA release causes vasoconstrictor effect through the activation of alpha-adrenoceptors [13]. The involvement of sensory innervation in the regulation of vascular tone depends on gender, physiological situation, and even rat strain [13±15].

In addition to the endothelial and neuronal factors, hormones also participate in the control of vascular function. Regarding sex hormones, cardioprotective effects have been reported in men and women [2, 16]. Previous studies from our group have shown that the loss of gonadal function in male and female rats increased the release and function of

vasoconstrictor prostanoids [17, 18], as well as the synthesis of ROS [13, 19, 20]. This accumulating production, when maintained for a significant amount of time, could lead to the development of cardiovascular diseases.

On the other hand, several studies have demonstrated the cardiovascular benefits of n-3 polyunsaturated fatty acids (n-3 PUFAs) [21, 22]. The most prominent *n*-3 fatty acids with demonstrated cardiovascular benefits are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are predominantly found in fish oils. The mechanisms by which these n-3 PUFAs decrease endothelial dysfunction involve lipid and prostanoid metabolism, leading to secondary favorable effects on blood pressure and thrombosis [23]. Additionally, we have reported the beneficial effect of a DHA-supplemented diet of orchidectomized rats on aorta function [24].

Considering all of these data and taking into account that mesenteric circulation has an important participation in systemic blood pressure control, it would be important to study the influence of PUFAs, specifically DHA, to prevent the functional alterations of mesenteric arteries observed after deprivation of male sex hormones. Therefore, the aim of this work was to study how a DHA-supplemented diet influences the mesenteric artery vascular function from orchidectomized rats, by monitoring: (1) the basal production of NO, ROS and prostanoids, (3) the vasodilator response to acetylcholine (ACh), as well as the involvement of prostanoids and NO in this response (4) the vasoconstrictor response to electrical field stimulation (EFS); additionally, the effect of exogenous NA, and the NO donor, SNP were also analyzed.

Materials and Methods

Animals, diets and experimental groups

The protocol was approved by the Animal Ethics Committee of the Universidad Autónoma de Madrid (Ref. CEI-37-829) and procedures were performed according to the European Union directives 63/2010UE and Spanish regulation RD 53/2013.

Male Sprague-Dawley rats (6 months old) were obtained from the Animal Quarters of the Universidad Autónoma de Madrid and housed in the Animal Facility of the Universidad Autónoma de Madrid (Registration number ES-20079-0000097), under 12 h light/dark cycles and standard feeding with fodder and water *ad libitum*. After 1 week of adaptation animals were fed a maintenance diet for rodents (Global Diet 2014, Harlan Laboratories Inc. Indianapolis, Indiana, USA) supplemented with fat (5%). The control group was supplemented with sunflower oil (5%) and the DHA group with 4.5% Marinol C-38 (Lipid Nutrition) and adjusted to 5% with sunflower oil. Nutrient content and energy distribution of each diet is summarized in Table 1. After 2 weeks on the diet, animals were divided into two groups: control and orchidectomized males. Male sex hormone deprivation was induced by orchidectomy at 18 weeks of age under anesthesia by isofluorane inhalation. Rats were treated with 0.30 mg/Kg meloxicam SC (Metacam 5 mg/ml; Boehringer-Ingelheim) immediately after surgery and with 50 mg/Kg ibuprofen, via oral administration for 4 days. Animals were maintained under experimental diets for six more weeks. At the end of the treatment, rats were sacrificed by CO_2 inhalation and decapitation. The observation of seminal vesicles atrophy confirmed successful surgery. The mesenteric artery was carefully dissected out, cleaned of connective tissue and placed in Krebs-Henseleit solution (KHS) (containing, in mM: NaCl 115, $CaCl_2$ 2.5, KCl 4.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11,1, Na_2 EDTA 0.03) at 4° C.

Table 1. Nutrient and energy content of experimental diets.

	Control Diet	DHA Diet
Carbohydrates (g/100g)	59.84	59.84
Protein (g/100g)	14.39	14.39
Total Fat (g/100g)	9.19	9.19
DHA + EPA		2.01
Energy (kcal/100g)	271.78	271.78

Blood pressure measurement

Systolic blood pressure was indirectly measured in awake animals by the tail-cuff method [17, 25] before and after the treatment using a Letica Digital Pressure Meter LE5000 (Barcelona, Spain).

Release of prostanoids

After a stabilization period in KHS at 37° C for 30 minutes (pH 7.4), mesenteric rings from each group of rats was followed by 2 wash periods of 10 min using 0.2 mL of KHS. Once fresh KHS was replaced, after a period of 10 min and the medium was collected and stored at -80ÊC until used. Production of TXA2, PGI2 and PGE2, were monitored by measuring their stable metabolite TXB2, 6-keto-PGF1α, and PGE2, respectively, using the respective enzyme immunoassay kit (Cayman Chemical). Results were expressed as pg prostanoid/mL per mg of tissue.

Production of nitric oxide

The fluorescent probe 4,5-diaminofluorescein was used to specifically evaluate NO production. Briefly, mesenteric segments from control, orchidectomized and orchidectomized with DHA diet groups were cryoprotected with 30% w/v sucrose in PBS, frozen and stored at -80ÊC. After a washing period with PBS, the artery segments were opened to uncover the artery lumen to allow a better penetrance of the probe. Then the segments were immersed in 4- (2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (in mM: NaCl 119, HEPES 20, $CaCl_2$ 1.2, KCl 4.6, KH_2PO_4 0.4, $MgSO_4$ 1, $NaHCO_3$ 5, glucose 5.5, $Na_2H_2PO_4$ 0.15; pH 7.4) containing 4,5-diaminofluorescein (0.5 µM), and incubated in a light-protected, humidified chamber at 37° C for 45 min. Then, the segments were mounted on glass slides and imaged on a confocal microscope. Images were obtained with a LEICA (TCS ST2 DM IRE2) laser scanning confocal microscope (excitation 495 nm, emission 515 nm). Laser and image settings were unchanged for the acquisition of images from the three groups of rats. The photomicrographs show the intensity and location of 4,5-diaminofluorescein, which reflects NO production, so that comparison of these groups could be made. To analyze fluorescence intensity, the ImageJ Analysis Software (National Institutes of Health) was used. The amount of NO released was expressed as arbitrary units.

Detection of superoxide anion

The fluorescent probe, hydroethidine, was used to evaluate superoxide anion levels *in situ*, as previously described [13, 26]. Mesenteric segments from the three groups were cryoprotected with 30% (w/v) sucrose in PBS, frozen and stored at -80° C. After a washing period with PBS, the artery segments were opened to uncover the artery lumen to allow for a better penetration of the probe. Then, the segments were immersed in HEPES buffer containing hydroethidine (5 µM), and incubated in a light-protected, humidified chamber at 37° C for 30 min. Segments were mounted on glass slides and imaged on a confocal microscope. Images were obtained with a LEICA (TCS ST2 DM IRE2) laser scanning confocal microscope (excitation 488 nm, emission 610 nm). Laser and image settings were unchanged for the acquisition of images from the three groups of rats. The photomicrographs show the intensity and location of hydroethidine, which reflects superoxide production, so that comparison of these groups could be made. To analyze fluorescence intensity, the ImageJ Analysis Software (National Institutes of Health) was used. The amount of superoxide formation was expressed as arbitrary units.

Vascular reactivity

The method used for isometric tension recording has been previously described [26, 27]. In summary, mesenteric artery segments were suspended in an organ bath containing 5 mL of KHS at 37° C, continuously bubbled with 95% O_2 -5% CO2 mixtures (pH 7.4). Two parallel stainless steel pins were introduced through the lumen of the vascular segment: one fixed to the bath wall and the other connected to a force transducer (Grass FTO3C; Grass Instruments Co., Quincy, MA, USA); this in turn was connected to a model 7D Grass polygraph. The activity was reflected in a computer through a computer program (eDAQ Software). The segments were subjected to a tension of 0.5 g which was re-adjusted every 15 min during a 90 min equilibration period before drug administration. After this, the vessels were exposed to KCl (75 mM) to check their functional integrity. After a washout period the viability of vascular endothelium was tested by the ability of 10 µM ACh to relax pre-contracted segments with 0.1 µM NA.

To determine the participation of innervation in the regulation of vascular tone in mesenteric artery, frequency-response curves to EFS were performed. The parameters used for EFS were 200 mA, 0.3 ms, 1±16 Hz, for 30 s with an interval of 1 min between each stimulus, the time required to recover basal tone. Since NO neurotransmitter and NA play key

roles in the mesenteric artery, concentration-response curves to NA (1 nM-10 µM) and the NO donor, SNP (1 nM-10 µM) were performed.

Concentration-response curves to ACh (0.1 nM-10 µM) were performed in NA (0.1 µM) pre-contracted mesenteric artery rings from of the three groups of rats. To analyze the participation of NO and prostanoids on the ACh-induced response, the NO synthase inhibitor L-NAME (0.1 mM) or the nonselective inhibitor of COX-1/2, indomethacin (Indo, 10 µM), were added to the bath 30 minutes before performing the curve.

Drugs

Drugs used were: L-NA hydrochloride, ACh chloride, L-NAME hydrochloride, indomethacin, potassium chloride, SNP (Sigma-Aldrich). Stock solutions (10 mM) of drugs were prepared in distilled water, except for NA which was dissolved in NaCl (0.9%)-ascorbic acid (0.01% w/v) solution, indomethacin in 1.5 mM $NaHCO_3$ in dimethylsulfoxide. These solutions were maintained at -20° C and appropriate dilutions were made in KHS on the day of the experiment.

Statistical analysis

Results are given as mean ± SEM (Standard Error of the Mean). The relaxation induced by ACh was expressed as a percentage of initial contraction elicited by NA. Statistical analysis was performed by comparing the curve obtained in the presence of the different substances with the control curve by means of two-way analysis of variance (ANOVA). For blood pressure, body weight, prostanoids, NO, and superoxide production, statistical analysis was done using Student's *t*-test for unpaired experiments. A *p* value of less than 0.05 was considered significant.

Results

Animal weight and systolic blood pressure

Pre-diet body weight and blood pressure measures from control, orchidectomized and orchidectomized with the DHA-supplemented diet were not significantly modified. Six weeks postdiet all groups increased body weight to a similar extent and blood pressure did not show significant changes. Results already published [24].

Production of nitric oxide

The orchidectomized group showed a decrease in the fluorescence emitted by 4,5-diaminofluorescein after incubation in the mesenteric tissue with respect to arteries from control rats. DHA-supplemented diet restored the fluorescence levels reduced by orchiectomy similarly to values found in control animals (Fig 1A).

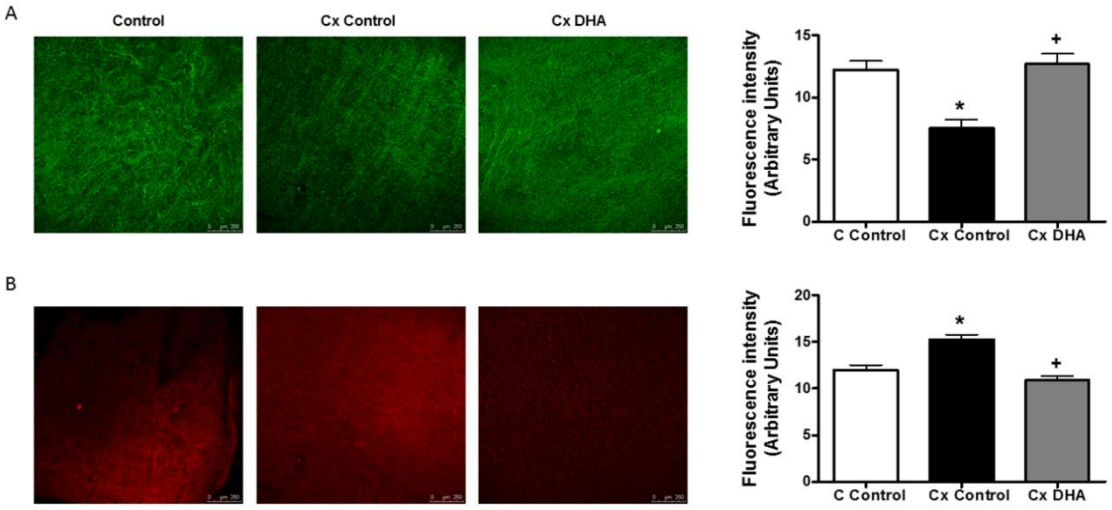

Fig 1. Effect of orchidectomy and DHA supplemented diet in endothelial production of NO and superoxide in rat mesenteric arteries. Confocal micrographs showing *in situ* detection of NO (A) or superoxide anion (B) in mesenteric artery segments from control (C) and orchidectomized (Cx) rats fed with a control diet and from Cx rats fed with a DHA-supplemented diet (Cx DHA). The sections shown are typical preparations from five rats. Quantitative analysis of fluorescence is also shown. Values are means ± SEMs, n = 5, **P* < 0.001 compared with control animals; *+P* < 0.002 compared with Cx rats control diet.

Detection of superoxide anion

HE fluorescence levels showed an increase in orchidectomized rat vessels, compared to the ones from control group. The DHA-supplemented diet decreased these levels on the orchiectomized rats (Fig 1B).

Release of prostanoids

Orchidectomy increased the basal release of TXB_2 and PGE_2 (Fig 2A and 2B). However, orchidectomy did not statistically modify the release of 6-keto-$PGF_{1\alpha}$ (Fig 2C). DHA supplemented diet decreased the release of the three prostanoids analyzed (Fig 2).

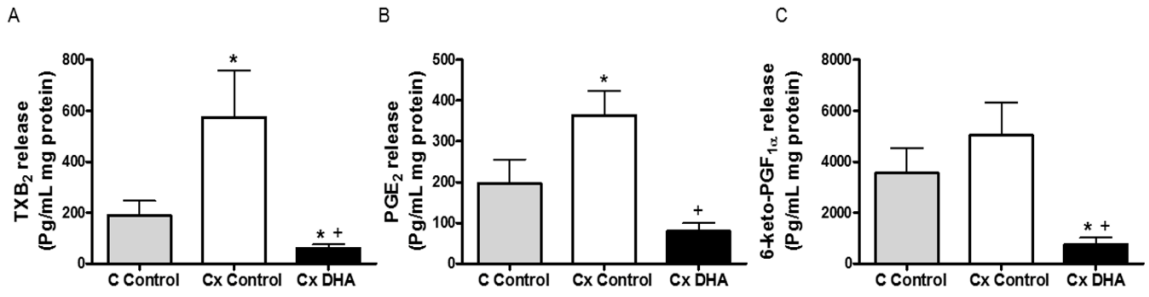

Fig 2. Effect of orchidectomy and DHA supplemented diet on prostanoid basal release in rat mesenteric arteries. Release of thromboxane B_2 (TXB_2), prostaglandin E_2 (PGE_2) and PGI_2 (panels A-C) in mesenteric artery from control (C) and orchidectomized (Cx) rats fed with a control diet and from Cx rats fed with a DHA-supplemented diet (Cx DHA). Values are means ± SEMs. n = 4±8; **p* <0.05 compared to arteries from control group, +*p <0.05* compared with arteries from Cx Control group.

Vascular reactivity

Mesenteric segments from all three groups contracted similarly to 75 mM KCl (C Control 1069 ± 78 mg; Cx Control 1291 ± 124 mg; Cx DHA 1199 ± 79 mg; $p > 0.05$).

Orchidectomy decreased the EFS-induced contraction which was reversed by the DHA supplemented diet (Fig 3). The contractile response induced by exogenous NA (10 nM- 10 µM) was similar in vessels from all three groups of rats (Fig 4).

The relaxation induced by SNP was analyzed in segments previously pre-contracted with NA (1 µM). Orchidectomy significantly increased the vasodilator response in segments from orchidectomized rats (ANOVA, $p < 0.001$, Fig 5). This response was not modified by DHA supplemented diet (Fig 5).

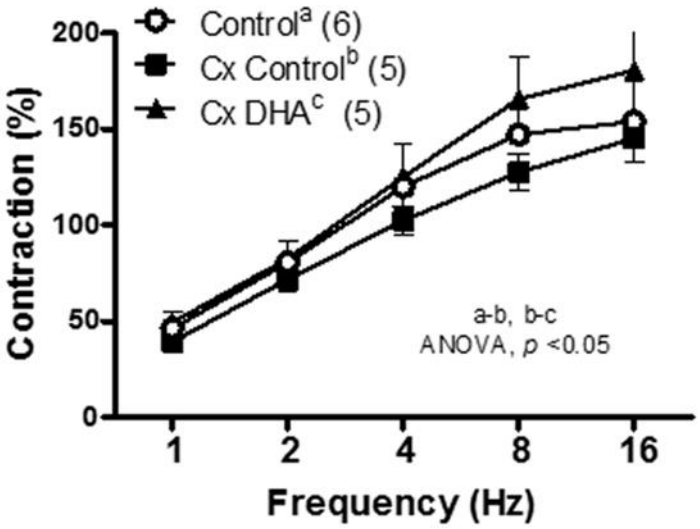

Fig 3. Effect of orchidectomy and DHA supplemented diet in the EFS-induced response in rat mesenteric arteries. Effect of EFS contractile response in mesenteric artery segments of male rats from control rats with control diet (A), orchidectomized rats with control diet (B) and orchidectomized rats with DHAsupplemented diet (C). Results (mean ± SEM) are expressed as percentage of contraction elicited by 75 mM KCl. Number of animals indicated in parenthesis

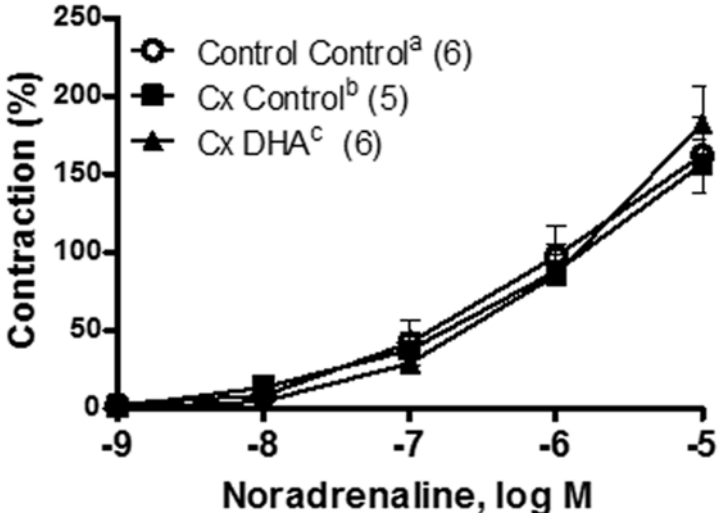

Fig 4. Effect of orchidectomy and DHA supplemented diet in the contractile response to NA curves in rat mesenteric arteries. Noradrenaline contractile response in mesenteric artery segments from control rats with control diet (A), orchidectomized rats with control diet (B) and orchidectomized rats with DHA-supplemented diet (C). Results (mean ± SEM) expressed as percentage of contraction elicited by 75 mM KCl. Number of animals indicated in parenthesis.

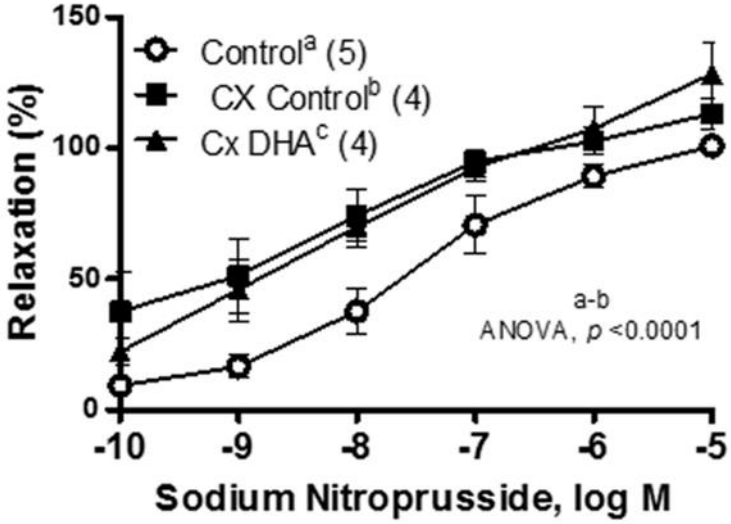

Fig 5. Effect of orchidectomy and DHA supplemented diet in the vasodilator response to SNP curves in rat mesenteric arteries. Orchidectomy and DHA supplemented diet influence on the vasodilator response to sodium nitroprusside in mesenteric artery segments of male rats, in control rats with control diet (A), orchidectomized rats with control diet (B) and orchidectomized rats with DHA-supplemented diet (C). Results (mean ± SEM) are expressed as percentage of inhibition of precontraction elicited by 1 µM noradrenaline. Number of animals indicated in parenthesis.

The vasodilator response induced by ACh (0.1 nMÐ10 µM) in segments pre-contracted with NA (1 µM) was not modified by orchidectomy, while the DHA supplemented diet increased this response (ANOVA, $p < 0.01$, Fig 6).

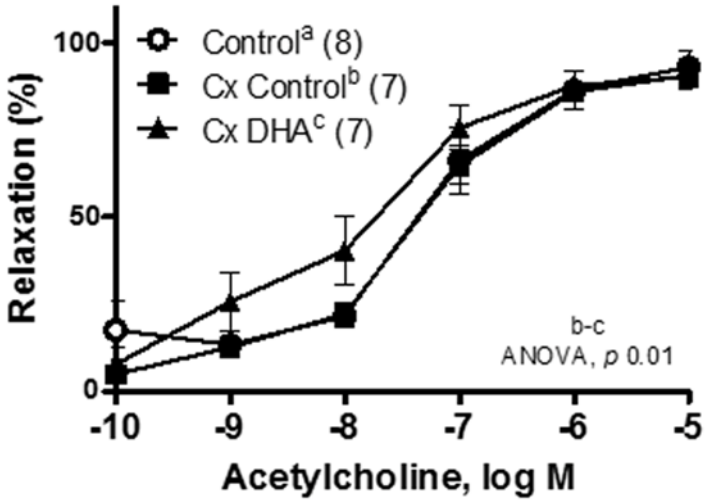

Fig 6. Effect of orchidectomy and DHA supplemented diet in the vasodilator response to ACh curves in rat mesenteric arteries. Concentration-response curves to acetylcholine in mesenteric artery segments from control (C) and orchidectomized (Cx) rats fed with a control diet and from Cx rats fed with a DHAsupplemented diet (Cx DHA). Results (means + SEMs) are expressed as percentage of inhibition of the contraction induced by 1 µMnoradrenaline. The number of animals is indicated in parenthesis.

The effect of castration and DHA supplemented diet in this stage on NO contribution was studied in presence of the NOS inhibitor, L-NAME, which decreased the ACh induced response in arteries from the control group (ANOVA, $p < 0.001$, Fig 7A), did not modify those of orchidectomized rats (ANOVA, $p > 0.05$, Fig 7B), whereas it decreased Cx DHA group (ANOVA, $p<0.0001$, Fig 7C). Incubation with the COX inhibitor, indomethacin (10 µM), tended to increase the ACh-induced response in control arterial segments (ANOVA, $p = 0.057$ 7-A), while increased the vasorelaxation in the orchidectomized group (ANOVA, $p = 0.004$, Fig 7B), and decreased it in Cx DHA group (ANOVA, $p = 0.02$, Fig 7C).

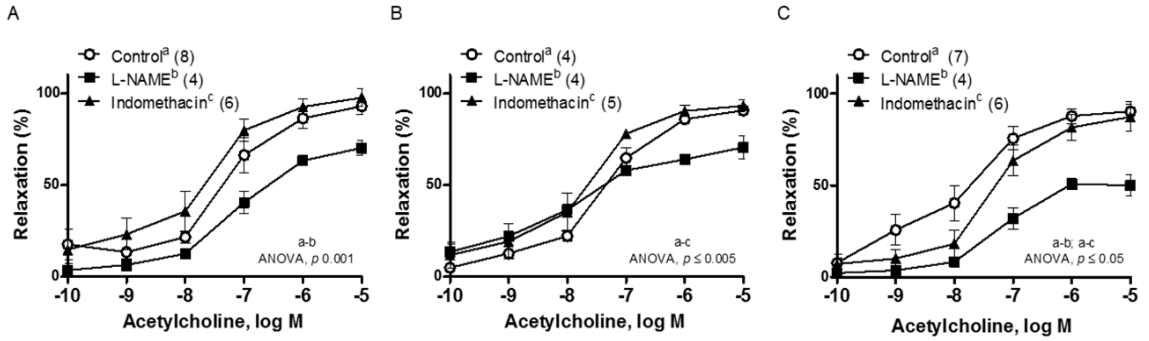

Fig 7. Effect of L-NAME and indomethacin in the ACh-induced vasodilator response in rat mesenteric arteries. Effect of L-NAME (0.1 mM) or indomethacin (Indo, 10 µM) on the concentration response curves to acetylcholine in the NA-pre-contracted mesenteric artery segments from control rats with control diet (A), orchidectomized rats with control diet (B) and orchidectomized rats with DHA-supplemented diet (C). Results (means ± SEMs) are expressed as percentage of inhibition of the contraction induced by 1 µM noradrenaline. The number of animals is indicated in parenthesis.

Discussion

There is compelling evidence which demonstrates the beneficial effect of n-3 PUFAs in vascular dysfunction for the prevention and treatment of cardiovascular disorders [21, 22]. Although the relationship between decreased levels of sex hormones and increased incidence of cardiovascular disease is established [28, 29], to date there are no studies examining the effect of diet supplemented with DHA when gonadal function is lost. Recently, we have reported positive modifications induced by a DHA-supplemented diet on lipid profile, redox status and vasodilator function of aorta from orchidectomized rats [24]. Taking into account that the mesenteric vascular bed importantly contributes to the control of blood pressure, in the present study we have focused on analyzing the effect of DHA-diet in the vascular function of rat mesenteric artery from orchidectomized rats.

We previously published that the orchidectomy maintained for 5 months induces an increase on prostanoid release in mesenteric artery [30, 31] and aorta [17, 25], due to an oxidative stress augmentation. The results now presented are in agreement with these previous findings, since TXA2, PGI2, and PGE2 are increased from the 6 week post-orchidectomy, as observed in aorta [24] and in mesenteric artery for the case of TXA2 [32]. However, DHA-supplemented diet decreased prostanoids release near to the control group levels. According with this data, the consumption of omega-3 fatty acid has been reported to inhibit the nuclear factor kappa B activation and of COX-2 expression [33], and to decrease the production of prostanoids [34]. Previous publications demonstrate that DHA competes with other PUFAs, such as the arachidonic acid, resulting in a decrease of prostanoid synthesis [35] and in an anti-inflammatory effect.

NO release was decreased by castration, as reported earlier in aortas from 6 weeks postorchidectomized rats [32, 24]. The influence of the DHA-supplemented diet, showed a restoration in NO release levels, closely to the ones from the control group, as previously found in aorta [24]. However, DHA has been reported not to modify the release of NO in aged [36] and hypertensive [37] rats, indicating that the choice of the experimental model is crucial.

NO bioavailability is strongly determined by the level of oxidative stress. It is already known that the loss of gonadal function increases the production of ROS [13, 20], like superoxide anion, a source of many other reactive oxygen intermediates, which quench NO. The results obtained in the present study show increased superoxide anion production in mesenteric artery from orchidectomized rats, which is in agreement with previous publications [13, 19], and that demonstrate the beneficial effects from male sex hormones in vascular function. Supplementation with the DHA diet diminished the superoxide anion levels near to

control levels, similar to those reported in the aorta of these animals [24]. In line with these results, is the ability of n-3 PUFAs to decrease superoxide anion production in mouse aorta [38] and human fibroblasts [39]. In addition, DHA has been reported to decrease oxidative stress in vascular [40], nervous [41] and immune [42] systems. All these data demonstrate the antioxidant properties of DHA in situations where oxidative stress is increased.

Taking into account that: i) NO, prostanoids and ROS are able to modulate the vascular tone, ii) the DHA-supplement diet restores the altered production of these factors in orchidectomized rats, and iii) the important contribution of mesenteric artery in the control of systemic vascular resistance, the vasoconstrictor response mediated by the neurotransmitters released after EFS and the endothelium-dependent vasodilator response were analyzed.

We observed that orchidectomy decreased the EFS-induced contraction, which is in agreement with that observed in mesenteric artery from 5 months-post-orchidectomized rats [13] in which the release of NA was not modified [18]. DHA-supplemented diet reversed the reduction in EFS-induced contractile response caused by orchidectomy. These actions seem to be specific on EFS-induced contraction since the contraction induced by 75 mM of KCl was not modified by the orchidectomy or DHA-diet. In this regard, the contractile response induced by potassium depolarization was not altered by a DHA diet in hypertensive rats [37]. Although, we have previously reported that the orchidectomy did not modify the release of neuronal NO [13] and NA [18], it would have been desirable to determine the neurogenic release of NA and NO. The authors acknowledge this limitation of the current study and, because of the intriguing and complex interactions among the different factors, this issue will be addressed in future studies. Nevertheless, it has been reported that DHA contained in fish oil did not significantly alter neuronal and cardiovascular control in normotensive and prehypertensive humans [43], while eicosapentanoic acid supplementation reduced cardiac noradrenaline concentration in diabetic rats [44], indicating that the cardiovascular benefits of omega-3 depend on the initial stage of the pathology. Since 5 months post-orchidectomy modified the vasomotor responses induced by NO and NA, these responses were analyzed in mesenteric arteries after 6 weeks post-orchidectomy fed control and DHA-supplemented diet. Orchidectomy did not produce any relevant influence on NA-induced contractile response, suggesting that this castration period does not interfere with the response once released the neurotransmitter, which is consistent with some publications [45]. However, it contrasts with other studies in which orchidectomy decreased [13, 46] or increased [47] NA-induced contractile response. These variations might be attributed to castration and/or the animal model employed as mentioned above. Additionally, the DHA-supplemented diet did not influence NA sensibility in smooth muscle cells, since its response was similar to that produced

by orchidectomy. This result is in agreement with studies describing no modification by DHA on the contractile response after alpha-adrenoceptors activation [48, 49]. Regarding the vasodilator effect of NO, the SNP-induced response was analyzed. The vasodilator response was significantly increased by orchidectomy, which agrees with reports on mesenteric arteries of rats after 5 months post-orchidectomy [31]. This response was not modified by the DHA supplemented diet, meaning that PUFAs have no effect on NO sensitivity in smooth muscle cell, as it was found in aortic segments [24] as published earlier [36]. Therefore, one possible explanation for the recovering of the decreased EFS-induced response in orchidectomized rats fed with DHA, could be the decline in the oxidative stress and, in turn, the decreased vasodilator effect from the NO-derived products [13]. However, the participation of different mediators cannot be discarded, which would be the goal of future research.

As stated before, the endothelium-dependent relaxation was also studied. In this regard, the ACh-induced response was unmodified by orchidectomy, as previously found in the aorta of these animals [24]. On the other hand, the diet supplemented with DHA significantly increased the endothelium dependent relaxation, caused by reduced oxidative stress, which increases NO bioavailability, as demonstrated by the levels of O_2^- and NO found in these arteries, and in aorta from a previous study [24].

To associate the participation of endothelial NO in the ACh- induced response, it was analyzed by using L-NAME, a NOS inhibitor. The decreased vasodilator response induced by ACh in the presence of L-NAME was grater in the rats fed with the DHA supplemented diet than in control and orquidectomized groups, evidencing that DHA increases NO participation. This response can be related to an increase of eNOS activation [50] aside from the increase in NO bioavailability, through the reduction of superoxide anion formation. The lack of change in the response from the orchidectomized group indicates a reduction in the participation of NO and the involvement from other vasodilator mechanisms apart from NO, such as prostanoids, when this is inhibited. Therefore, the role of prostanoids in ACh response was elucidated by incubation with the COX inhibitor, indomethacin. The increased response in orchidectomy suggests the involvement of vasoconstrictor prostanoids. For this reason, when prostanoids are removed by indomethacin the relaxation to ACh is greater, although the participation of other vasodilator factors cannot be discarded. However, in the DHA group vasodilator prostanoids are predominant, so ACh induced relaxation is smaller in the presence of indomethacin which suggests that the diet does improve more vasodilator than vasoconstrictor prostanoids. This is contrary to results found in aorta, where there was no modification of this response [24], indicating that the vascular effect of DHA in the relaxing response mediated by prostanoids depends on the vascular bed. It has been reported that

when prostanoid synthesis is inhibited, NO synthesis is modified [25] and viceversa; also, other factors could be working to compensate the loss of prostanoids [18].

In summary, orchidectomy was associated with endothelial dysfunction of mesenteric artery caused from increased oxidative stress, by means of an augmented formation of prostanoids and superoxide production, and a reduction in endothelial NO formation. Also, the neurogenic response was decreased. The DHA-supplemented diet decreased prostanoid and superoxide levels, at the same time that increased NO formation and bioavailability in orchidectomized rats, and recovered the neurogenic response that may account for a better regulation of vascular function. The results obtained in this study together with others previously reported [24] suggest that a DHA-supplemented diet may be helpful to treat cardiovascular disease, since this PUFA exerts beneficial effects, in addition to the lipid profile, on the function of both conduit and resistance arteries

Supporting Information

S1 File. Individual experimental data points for Fig 1A and 1B. (XLSX)

S2 File. Individual experimental data points for Fig 2A, 2B and 2C. (XLSX)

S3 File. Individual experimental data points for Fig 3. (XLSX)

S4 File. Individual experimental data points for Fig 4. (XLSX)

S5 File. Individual experimental data points for Fig 5. (XLSX)

S6 File. Individual experimental data points for Fig 6. (XLSX)

S7 File. Individual experimental data points for Fig 7A, 7B and 7C. (XLSX)

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Publication 3:

Dietary docosahexaenoic acid supplementation prevents the formation of cholesterol oxidation products in arteries from orchidectomized rats

PLOS ONE

RESEARCH ARTICLE

Dietary docosahexaenoic acid supplementation prevents the formation of cholesterol oxidation products in arteries from orchidectomized rats

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Abstract

Testosterone deficiency has been correlated with increased cardiovascular diseases, which in turn has been associated with increased oxidative stress. Several studies have considered cholesterol oxidation products (COPs) as oxidative stress biomarkers, since some of them play pro-oxidant and pro-inflammatory roles. We have previously described the cardioprotective effects of a dosahexaenoic acid (DHA) supplemented diet on the aortic and mesenteric artery function of orchidectomized rats. The aim of this study was to investigate whether impaired gonadal function alters the formation of COPs, as well as the potential preventive role of a DHA-supplemented diet on that effect. For this purpose, aortic and mesenteric artery segments obtained from control and orchidectomized rats, fed with a standard or supplemented with DHA, were used. The content of the following COPs: 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 5,6α-epoxycholesterol, 5,6β-epoxycholesterol, cholestanetriol and 25-hydroxycholesterol, were analyzed by gas chromatography. The results showed that orchidectomy increased the formation of COPs in arteries from orchidectomized rats, which may participate in the orchidectomy-induced structural and functional vascular alterations already reported. The fact that the DHA-suplemented diet prevented the orchidectomy-induced COPs increase, confirms the cardiovascular protective actions of DHA, which could be of special relevance in mesenteric arterial bed, since it importantly controls the systemic vascular resistance.

Introduction

An important number of studies have shown a correlation between increased cardiovascular diseases with testosterone deficiency [1, 2]. It has been demonstrated that sex hormones regulate vascular function since sex hormones deprivation alters the release, function and cell signaling pathways of endothelial factors that could lead to vascular dysfunction. Thus, previous studies from our research group have shown that orchidectomy increased ROS production [3, 4] and prostanoids release [5-7], while the production of nitric oxide [8] and the antioxidant capacity [9-10] were reduced.

The increased oxidative stress has been associated to the development of cardiovascular diseasses [11, 12]. Thus, oxidative damage of cellular membranes and enzymes by reactive oxygen and nitrogen species (ROS/RNS) has been described in cardiovascular diseases [13].

Oxidative modification of cholesterol from cell membranes by ROS/RNS leads to the formation of cholesterol oxidation products (COPs), also called oxysterols. COPs can also be formed by enzymatic oxidation and/or by absorption from the diet [14-16]. Regardless of the source, COPs have the ability to induce disruption of fine structure, alteration of integrity, fluidity, and permeability, and loss of biomembrane functionality [17, 18].

Furthermore, oxysterols have been implicated in the pathogenesis of various diseases including cardiovascular diseases, cancer, neurological disorders, and aging [19-22]. COPs are able to modify low-density lipoproteins (LDL) and high-density lipoproteins (HDL) into pro-atherogenic and pro-inflammatory forms. Oxysterols can also trigger pro-oxidative, pro-inflammatory and cytotoxic reactions in the different cell types of the vascular wall, including endothelial cells [23], smooth muscle cells [24-25], fibroblasts [26], monocytes and macrophages [27]. Derived from such evidence, COPs have been proposed as potential biomarkers for non-invasive studies of oxidative stress *in vivo* [28].

Different studies have demonstrated that consumption of Omega-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) may reduce the risk of developing cardiovascular diseases [29, 30]. The anti-thrombotic, anti-inflammatory and vasoprotector effects of PUFAs on the cardiovascular system have been reported [31, 32]. Antioxidant propierties of DHA have been also demonstrated since it decreased ROS formation and bound to free radicals preventing tissue oxidation [33-35].

Since it was reported that the lack of sex hormones increases oxidative stress and vascular inflammation, deteriorating factors that could lead to the development of CVD, it is possible that formation of oxysterols in the arterial wall could be modified by the loss of

gonadal function. On the other hand, Omega-3 PUFAs may act as a cardioprotective treatment in the oxysterol formation induced by orchidectomy. Therefore, the purpose of the present study was to determine the effects of orchidectomy on the formation of the main oxysterols recognized as products of cholesterol autoxidation [36] (7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 5,6α-epoxycholesterol, 5,6β-epoxycholesterol, cholestanetriol and 25-hydroxycholesterol) in the arterial wall of the aorta and mesenteric artery, as well as the possible preventive effect of a DHA-supplemented diet on COPs formation.

Materials and Methods

Animals, diets and experimental groups.

Male Sprague-Dawley rats (18-week-old) were purchased from Envigo (Mexico City). Rats were housed in stainless steel cages in a temperature-controlled (23 ± 2 °C) room, under 12-hour light/dark cycles and standard feeding with fodder and water *ad libitum*. After 4 weeks of adaptation, animals were fed a maintenance diet for rodents (2018S Teklad global 18% protein rodent diets, Envigo, Madison, WI, USA) supplemented with fat (5%). The controls-diet groups were supplemented with sunflower oil (5%) and the DHA groups with 4.7% MEG-3® (Ocean Nutrition Canada Ltd., Dartmouth, NS, CA) and adjusted to 5% with sunflower oil. Nutrient content and energy distribution of each diet is summarized in Table 1. After 2 weeks on the diet, animals were divided into two groups: control and orchidectomized males. Male sex hormone deprivation was induced by orchidectomy at 24 weeks-old under injectable anesthesia with Ketamine-Xylazine (80 mg/kg ket plus 10 mg/kg xil; IP). Rats were treated with 2 mg/kg meloxicam SC (Metacam 5 mg/mL; Boehringer Ingelheim) and with 50 mg/kg ibuprofen immediately after surgery, via IP administration for 5 days. The observation of seminal vesicles atrophy confirmed successful surgery. Animals were maintained under the experimental diets for six more weeks. At the end of the treatment, rats were sacrificed by ether inhalation and decapitation. The mesenteric artery and the aorta were carefully dissected out, cleaned of connective tissue and placed in Krebs-Henseleit solution (KHS) containing, in mmol/L: NaCl 115, $CaCl_2$ 2.5, KCl 4.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.1, Na_2 EDTA 0.03 at 4 °C. The investigation was performed in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the USA National Institutes of Health (NIH publication No. 85.23 revised 1985), and approved by the Ethical Committee of the *Universidad Autónoma de Madrid* according to directives of the Ministerio de Agricultura, Pesca y Alimentación of Spain (PROEX 202/16).

Table 1. Nutrient and energy content of experimental diets.

Macronutrient	Control diet (g/100g)	DHA diet (g/100g)
Crude protein	18.6	18.6
Fat	11.2	11.2
Carbohydrate (available)	44.2	44.2
Crude Fiber	3.5	3.5
Neutral Detergent Fiber	14.7	14.7
Ash	5.3	5.3
Sunflower oil	5.0	0.3
DHA+EPA	0	4.7
Energy Density kcal/100g	310.0	310.0

Determination of Cholesterol and COPs in arterial Tissue Samples

Lipids extraction

Two aortic and two mesenteric rings (*ca*. 3 mm length) from each animal (from the all experimental group of rats) were individually weighed, thawed and subjected to total lipid extraction, as described by Folch *et al*. [37] with some modification [16]. Briefly, arterial rings were homogenized in 1 mL of PBS at 4 °C containing BHT (0.05 %). Each arterial homogenate was mixed with 16 mL of the Folch solution (chloroform-methanol 2:1 v/v) and 0.01% BHT. Then, 3 mL of NaCl aqueous solution (0.73 %) were added. The resulting mixture was separated by centrifugation. The upper phase was discarded and the lipids were collected from the organic layer (lower phase). A mixture of chloroform-methanol-0.73% NaCl (3:48:47 v/v/v, 7.5 mL) was added to the recovered phase and then left at 4 °C for 4 h to allow separation. The lower phase was recovered and then filtered through anhydrous sodium sulfate. Subsequently, the extract was resuspended in chloroform with BHT (0.02 %) and frozen at -20 ° C until used.

Saponification

Cold saponification was performed according to the method of Menéndez-Carreño *et al.* [38] to remove glycerides, free fatty acids and water-soluble impurities, as well as to release the esterified COPs. For sample preparation, each lipidic extract from the arterial tissue samples was added with 13 µg of the internal standard, 19-hydroxycholesterol (19-OH) (disolved in *n*-hexane:isopropanol, 4:1 v/v) for COPs quantification and with 1 mg of 5α-colestane (disolved in *n*-hexane: isopropanol, 4:1 v/v) as an internal standard for the

quantification of cholesterol. The mixture was then dried under stream of nitrogen and mixed with 3 mL of a KOH solution in methanol (4 mol/L or 4 N) with BHT (0.05 %), wrapped with aluminium foil and subjected to orbital shaking (300 rpm) at room temperature for 18 h to produce saponification.

For extraction of the unsaponifiable matter, 10 mL of dichloromethane and 5 mL of citric acid (0.1% in double-distilled water) were added to each sample, and mixed vigorously in a vortex. The diethyl ether fraction was then separated by centrifugation. The aqueous layer (supernatant) was discarded and the organic phase was washed with portions of 5 mL citric acid (0.1%, v/v) solution until clear. The organic phase from the samples was dried over anhydrous sodium sulfate. The organic solvent was evaporated with a rotary evaporator at 50 °C to remove the dichloromethane. The unsaponifiable extract was dissolved in diethyl ether in a conical vial and dried under nitrogen flow for the subsequent quantification of cholesterol and COPs.

Analysis of total cholesterol by gas chromatography

For cholesterol determination, 10% of the unsaponificable matter solution was dried under nitrogen flow and subjected to silylation according to Sweeley *et al*. [39]. A pyridine:hexamethyldisilazane:trimethylchlorosilane (5:2:1, v/v/v) mixture was added (0.3 mL) and heated at 40 °C for 20 min. The mixture was then dried under a stream of nitrogen and dissolved in 1 mL of *n*-hexane.

Analyses were performed on an Agilent 7820A gas chromatograph. 1 µL of sample was manually injected in split mode (1:1). Separation of the compounds was performed on a Perkin Elmer PE-5 capillary column (30 m x 0.32 mm d.i. x 1 µm film thickness coated with 5% -phenyl-methylpolysiloxane). The oven temperature was programmed from 280 °C to 300 °C at a rate of 10 °C/min, and mantained for 30 min. The injection and detection port temperatures were set at 325 °C. UHP nitrogen was used as the carrier gas at a rate of 1.5 mL/min.

Total cholesterol from the arterial tissue lipidic extracts was quantified by the internal standard method, using 5α-cholestane. Identification of the cholesterol peak in the samples was carried out comparing the retention times with that from the standard. Quantification of cholesterol was performed using a calibration curve.

Purification and derivatization of COPs

The remaining 90% of the unsaponificable matter were dried under nitrogen stream and re-suspended in 300 µL of *n*-hexane:ethyl acetate (95:5, v/v) and purified by NH_2 SPE cartridges, previously equilibrated with 3 mL *n*-hexane deied with anhydrous sodium sulfate at the bottom. The cartridge was eluted with the following solvent sequence: 6 mL of *n*-hexane:ethyl acetate (95:5, v/v), 10 mL of *n*-hexane:ethyl acetate (90:10, v/v) and 10 mL of acetone. The COPs were eluted from the NH_2 SPE cartridge with the acetone fraction according to the method of Rose-Sallin *et al.* [40]. The purified COPs were dried under nitrogen flow and silylated, according to the method of Sweeley *et al*. [39], and dissolved in 50 µL of *n*-hexane.

Analysis of COPs by gas chromatography

One microliter of the silylated COPs was manually injected into the GC under the same conditions used for the determination of total cholesterol. Total COPs were quantified using 19-hydroxycholesterol (19-OH) as internal standard. The COPs identification in the samples was performed by comparison of the retention times with those of the COPs standards and the quantification was performed by calibration curves, as it was done for cholesterol.

Reagents

All analytical grade solvents and reagents were supplied by Tecsiquim (Mexico City) and from Sigma-Aldrich (Mexico City). For identification and quantification of each COP the internal standards utilized were 19-hydroxycholesterol (internal standard for the quantification of COPs), 5*α*-cholestane (internal quantification standard for cholesterol), Cholest-5-en-3*β*-ol (cholesterol), cholest-5-en-3β-ol-7-one (7-ketocholesterol), 5α,6α-epoxycholestane-3β-ol (5,6α-epoxycholesterol), 5β,6β-epoxycholestane-3β-ol (5,6β-epoxycholesterol), cholestane-3β,5α,6β-triol (cholestanetriol), cholest-5-en-3β,7β-diol (7β-hydroxycholesterol), and cholest-5-en-3β,25-diol (25-hydroxycholesterol). Cholest-5-en-3*α*,7*α*-diol (7*α*-hydroxycholesterol) standard was supplied by Steraloids (Newport, CT, USA).

Solid-phase extraction (SPE-NH_2) cartridges (500 mg amino-propyl stationary phase/3 mL) were purchased from Phenomenex (Grace Discovery Sciences, Deerfield, IL, USA). The silylation mixture was prepared with dried pyridine, hexamethyldisilazane and trimethylchlorosilane, from Sigma.

Statistical analysis

All values are expressed as the mean ± SEM (Standard Error of the Mean) of the five animals used. For each value, the mean of the cholesterol and COPs quantification -obtained in each of the two arteries from the same animal- was calculated. Statistical analysis of COPs concentration was performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. For body weight, statistical analysis was done using paired Student's *t*-test. A *p* value of less than 0.05 was considered significant. Data were analyzed using the GraphPad Software (San diego, CA, USA).

Results

Animal body weight

Animals remained healthy and behaved similarly well throughout the experiment. Before the experimental diets (control or DHA) were administered, body weight was evaluated in the four groups of animals, showing no statistical differences among the groups (Table 2). The average weight gain at the end of 8 weeks under the experimental diets was similar for all the experimental groups, which is consistent with our previously published data [9].

Table 2. **Body weight (g) in control (C) and orchidectomized (ORX) rats fed with a control or DHA-supplemented diet.**

	Body weight (g)	
Animal group	Before diet	After diet
C Control	418.5 ± 11	463 ± 10*
C DHA	385.1 ± 12	435 ± 19*
ORX Control	384.6 ± 13	437 ± 12*
ORX DHA	409.2 ± 16	423.4 ± 16*

Values are means ± SEMs. Number of animals per group, n = 5.
*Indicates differences with its respective groups before diets.

Cholesterol content in aorta

Orchidectomy significantly increased cholesterol levels in the aortic tissue compared to the control group fed with the control diet (Fig. 1). The DHA-supplemented diet caused a significant decrease in cholesterol levels in the aorta from orchidectomized animals. Samples from control rats fed with the DHA-supplemented diet showed similar levels to those found in the control rats fed with the control diet.

COPs content in aorta

Total COPs levels significantly increased in the orchidectomized group, and decreased in orchidectomized animals fed the DHA-supplemented diet (Fig. 1). DHA supplementation did not stastically modify total COPs content in control rats.

Consistent with the above trend, orchidectomy caused a significant increase in the concentration of the studied COPs in aortic segments: 7α-hydroxycholesterol (7α-OH), 7β-hydroxycholesterol (7β-OH), 7-ketocholesterol (7-KC), 5,6β-epoxycholesterol (5,6β-E), 5,6α-epoxycholesterol (5,6α-E), 25- hydroxycholesterol (25-OH), and cholestanetriol (CT) (Fig. 1).

The DHA-supplemented diet decreased COPs levels in the aortic tissue of orchidectomized rats, except 7β-OH which did not reach stastistical difference (Fig. 1). However, the DHA-diet to control animals tended to increase the concentration of all analyzed COPs.

The proportion in the concentrations of the analyzed COPs in relation to the total COPs was similar in the different groups included in this study. CT was the most abundant COP (*ca.* 30% from total COPs), followed by 7β-OH (*ca.* 20% from total COPs), 7α-OH (*ca*. 18%), 5,6β-E (*ca.* 12%). 7-KC, was higher in both orchidectomized groups (fed with control and DHA-suplemented diet) the ratio remained above the control groups by *ca.* 8-10%. The proportion of 5,6α-E was similar in all groups, being ca. 5% of total COPs. 25-OH could not be detected in the control rats fed the control diet and for the other groups this COP was detected in very small concentrations (≤1%).

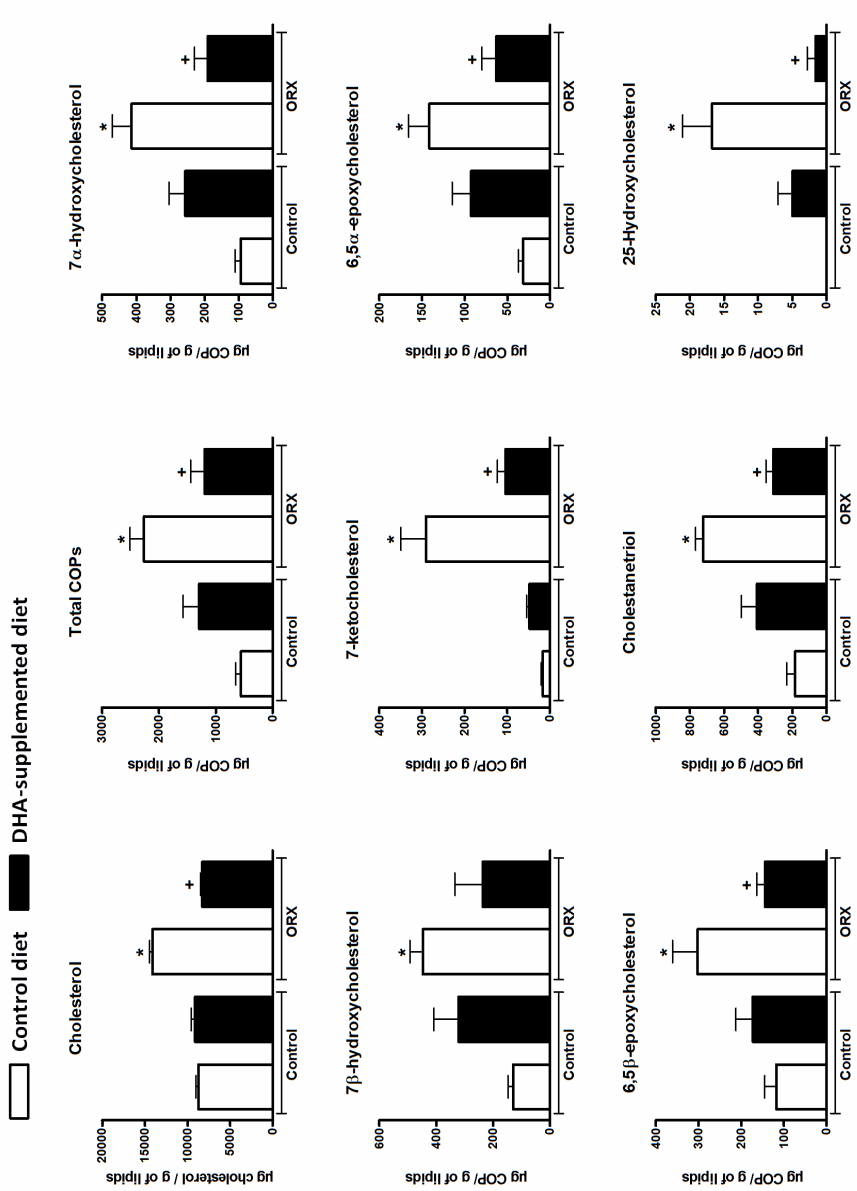

Fig. 1. Effect of orchidectomy and DHA suplementation on cholesterol and COPs content in rat aorta. Graphic representation of Chol and COPs (7α-OH, 7β-OH, 7-KC, 5,6β-E, 5,6α-E, 25-OH, CT and total COPs) concentration in μg/g of lipids in aortic rings from control (C) and orchidectomized (ORX) rats fed with a control or with a DHA-supplemented diet. Data were compared using one way ANOVA followed by Tukey's multiple comparison tests. Values are means ± SEMs. Number of animals, n = 5. *Indicates a $p < 0.05$ *vs* Control group fed control diet. $^{+}$Indicates a $p< 0.05$ *vs* orchidectomized rats fed control diet.

Cholesterol content in mesenteric artery

The content of cholesterol in mesenteric artery was significantly increased by orchidectomy and the DHA-supplemented diet significantly decreased those levels (Fig. 2). The DHA-diet to control rats did not statiscally modify the content of cholesterol in mesenteric artery tissue (Fig. 2).

COPs content in mesenteric artery

Similar results were observed for COPs content in mesenteric artery respect to those found in aorta from these animals. Total COPs content increased significantly in samples from orchidectomized animals, compared to the rest of the groups. The control animals fed with the control diet as well as those fed with the DHA-supplemented diet showed similar COPs levels. Again, DHA supplementation decreased COPs content in orchidectomized animals (Fig. 2).

Orchidectomy significantly increased 7α-OH, 7β-OH, 7-keto, 5,6β-E, 5,6α-E, 25-OH, and CT content (Fig. 2) compared to the rest of the groups included in the study. As observed in Fig. 2, 25-OH content from control rats fed with the control diet was not detected. The same ocurred with 7-KC, which was only detected in minimal amounts in the orchidectomized group, whereas it was practically absent from the mesenteric artery tissue in the other groups.

In the mesenteric arteries from the orchidectomized animals fed with the DHA supplemented diet the concentration of COPs decreased significantly, except for 7β-OH, similarly to that found in aortic tissue (Fig. 2). DHA supplementation in control animals tended to decrease the concentration of all the analyzed COPs (7α-OH, 7β-OH, 7-KC, 5,6β-E, 5,6α-E, 25-OH, and CT), unlike the observed in aorta.

Regarding the distribution of COPs content in mesenteric artery tissue, CT was the most abundant COP (*ca.* 30%) followed closely by the 7α-OH and 7β-OH, which showed similar proportions between the same groups, arround 20% of total COPs. Next in proportion is the 5,6β-E representing *ca.* 15% of total COPs, with a slightly higher proportion in the control group (19%). The 5,6α-E represents about 10% of the total COPs, dropping to 6% in the orchidectomized animals fed the DHA-supplemented diet. 25-OH was practically absent, since it could not be detected. In the case of 7-KC, it was only detected in the orchidectomized group at very low concentrations.

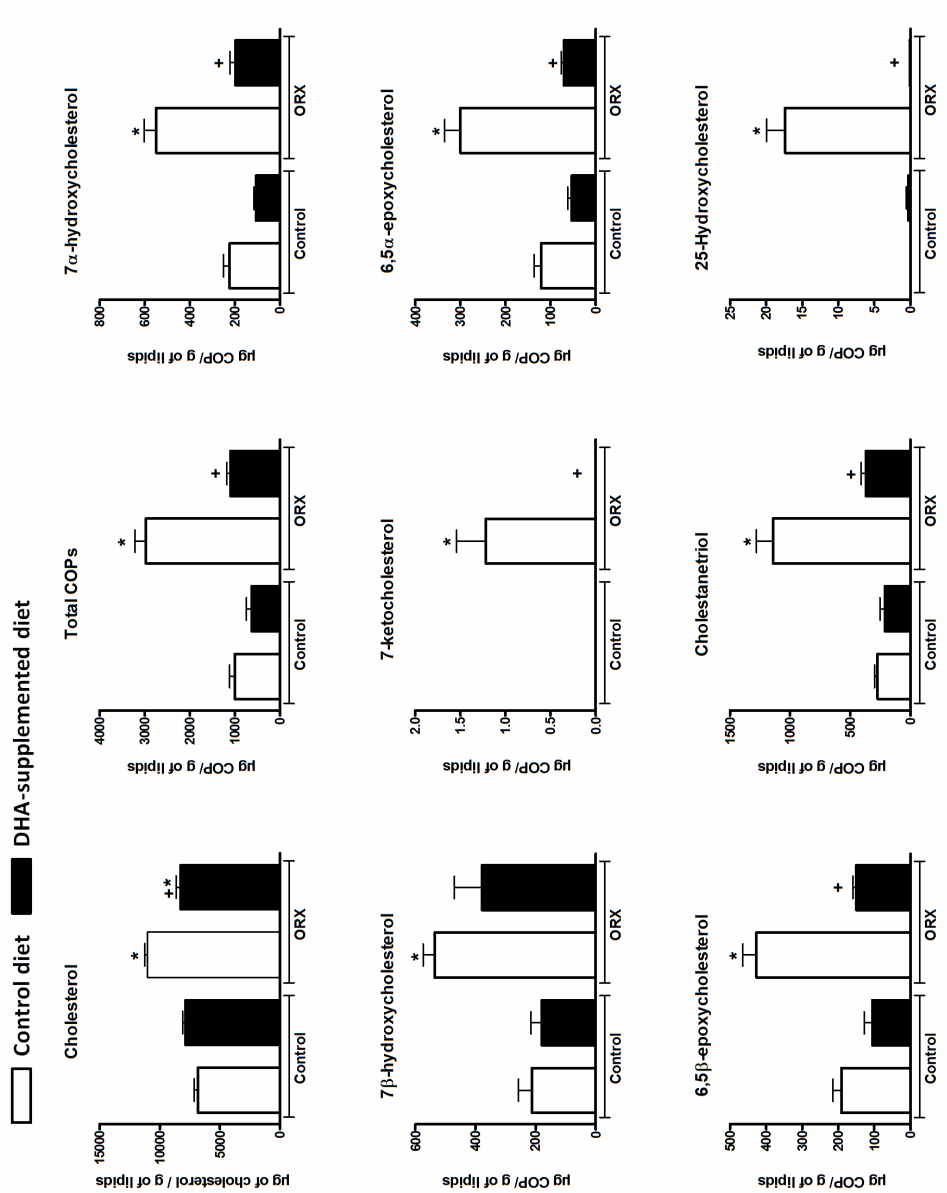

Fig. 2. Effect of orchidectomy and DHA suplementation on cholesterol and COPs content in rat mesenteric artery. Graphic representation of Chol and COPs (7α-OH, 7β-OH, 7-KC, 5,6β-E, 5,6α-E, 25-OH, CT and total COPs) concentration in μg/g of lipids in mesenteric artery rings from control (C) and orchidectomized (ORX) rats fed with a control or with a DHA-supplemented diet. Data were compared using one way ANOVA followed by Tukey`s multiple comparison tests. Values are means ± SEMs. Number of animals, n = 5. *Indicates a $p < 0.05$ vs. Control group fed control diet. $^{+}$Indicates a $p < 0.05$ *vs* orchidectomized rats fed control diet.

Discussion

This study shows, for the first time, that the loss of gonadal function induced an increase in the formation of COPs in both aorta and mesenteric artery wall. The preventive action of a DHA-supplemented diet on the orchidectomy-induced COPs formation is also demonstrated. These results are in agreement with data previously reported on both the effects of orchidectomy and the beneficial role of a DHA-supplemented diet.

Regarding the results specifically associated to orchidectomy, we have previously reported that the loss of gonadal function of rats induced an overproduction of ROS in aorta [3] and mesenteric artery [4], as well as an increase in the serum content of cholesterol, LDL-cholesterol and triglycerides [9]. It is known that the cholestesterol embeded on the lipid bilayer from the cell membranes is prone to oxidation by ROS [41, 42] which could explain the increased formation of COPs derived from autoxidation processes described in this work. However, oxidation during sample processing can not be ruled out, despite of the utilization of BHT (0.05 %). Likewise, it is important to consider that conversion to COPs other than those analyzed may exist. It is well known that the preferential site of oxidation of cholesterol by highly reactive species is at C7 having a relatively weak carbon-hydrogen bond. Moreover, the unique cholesterol double bond between carbons 5 and 6 comprises a vulnerable site for oxidation by free radicals. The most abundant non-enzymatic cholesterol oxidation products present in most tissues are 7α-OH, 7β-OH, 7-KC, and 5,6α-E and 5,6β-E, respectively [43, 44] induced by ROS and RNS [45], which is in line with the findings from the current study, except for 7-KC, which was only detected in mesenteric arteries from orchidectomized rats. CT is one of the most abundant oxysterols, derived from cholesterol by oxidation via formation of 5,6α-E and 5,6β-E as intermediates [46]. The very low detection leves of 25-OH may be attributed to its primarily enzymatic origin, since this oxysterol is generally not considered to be a significant autoxidation product of cholesterol. In addition, the reported levels of 25-OH in most tissues are extremely low [36]. Determination of COPs content can vary depending on the extraction and/or measurement methods, although very low COPs levels under physiological conditions have been reported [47]. In this sense, the 7-KC, 7α-OH and 5,6α-E content -referred to total cholesterol- reported in the present study were similar to those described in human aorta [48] and coronary arteries [49]. However, the relative7-KC, 7α-OH, 7β-OH, 5,6α-E and 5,6 β-E content found in our study are higher than those described in human aorta and carotid artery [49, 50].

On the other hand, COPs are known to trigger oxidative stress by increasing the generation of superoxide anion [51, 52], and down-regulating the expression/activation of Nrf2 [53] Consistent with these results, many studies have reported increased COPs levels in the membranes of cells subjected to oxidative stress, as it has been detected in patients with diabetes mellitus [54], hiperlipidemia [28], chronic inflammatory processes and chronic renal failure [55]. In this regard, the oxysterol clearance mechanisms are probably less efficient in those situations [44], as it was observed in orchidectomized rats in wich the antioxidant activity was decreased [9]. Zhang *et al*. [56] demonstrated that castration decreased enzimatic antioxidant activity (SOD and glutathione peroxidase) and increased malondialdehyde levels, an indicator of lipid peroxidation. In contrast, other studies found that castration did not increase lipid peroxidation [57]. These discrepancies could be attributed to differences in the animal model used, since the maintenance period of gonadectomy determines the induced alterations [8, 58]. However, it is important to emphasize that the experimental model used in this study is the same as that used in our two previous studies [9, 10] and provides relevant information about the key role of the COPs in the orchidectomy-induced modifications of factors, additional to ROS, which regulate vascular function.

It has been mentioned above that different cardiovascular risk factors, such as hypertriglyceridemia, hypertension, diabetes, obesity and overweight, have been associated to COPs content in human serum [15]. In agreement with these results, COPs induced increased blood pressure, serum triacylgycerols as well as body fat index in Wistar rats [59]. On the other hand, the induction of hypertension to rabbits by coarctation of the aorta showed an enhancement of COPs content in both plasma and aortic tissue 60]. In addition, Ares *et al*. [61] indicated that COPs promoted the stimulation of MAPK in human aortic smooth muscle cells, which could partially explain the activation of the MAPK sinaling pathway reported in hypertension after tyrosin kinase receptor transactivation [62]. In this regard, our group has described the activation of MAPK and AKt pathways in mesenteric arteries of orchidedctomized rats, in which the transactivation of EGFR was involved [8]. In this context, these results would indicate that the increase in the formation of COPs may participate in the orchidectomy-induced structural alterations that, in the long term, can lead to the development of hypertension, as it occurs in aged-orchidectomized rats.

Apart from these effects, COPs alter prostaglandin synthesis and stimulate platelet aggregation, an important process facilitating atherosclerosis and thrombosis [42]. Likewise, different COPs induced the expression of COX-2 [63] and the release of prostaglandin E_2 [64] and prostaglandin J_2 [65], considered as pro-inflammatory events. Accordingly, it has been described that orchidectomy also leads to a pro-inflammatory environment by increasing

prostanoid production in rat aorta [6, 8, 9] and mesenteric arteries [7, 8, 10] most likely as a consequence of increased oxidative stress produced during the experimental conditions.

Nitric oxide is also a critical molecule in vascular function, and the inhibition of endothelial NO-synthase by COPs has been described [52, 66], which is also in agreement with the decreased NO formation observed in aorta [9] and mesenteric artery [10] from orchidectomized rats. Although the decrease of NO production by COPs seems to be demonstrated, studies addressing the influence of COPs in the vascular function have shown contradictory results, since inhibition [67, 68] or no modification [69, 70] of endothelial-dependent vasodilation by 7-KC and 7α-OH-cholesterol have been reported. In our experimental model, orchidectomy did not alter the acetylcholine-induced response in aorta or mesenteric artery, since factor/mechanisms other than NO are simultaneously working to maintain a proper function [4, 7].

These results can be summarized in that orchidectomy induced an increase of the oxidative stress and anti-inflammatory mediators, and a deterioration of the lipid profile, which is accompanied by an increase in the formation of COPs. Interestingly, cholestanetriol -the most abundant COP in all samples, especially in arteries from orchidectomized rats- has been reported to be one of the most cytotoxic oxysterols in different cell lines such as rabbit aortic smooth muscle cells [71], mouse L cells [72], Chinese hamster V79 lung fibroblasts [26], human monocytic cells (U937), colonic adenocarcinoma cells (CaCo-2) and hepatoma liver cells (HepG2) [73]. However, it is important to point out the cytotoxic effects reported for different COPs other than cholestanetriol, also in different cell lines, related to vascular and nervous systems that contribute to the pathogenesis of cardiovascular [52, 74] and neurodegenerative [19, 20] diseases.

It has been suggested that generation of some oxysterols can be reduced in the presence of antioxidants [43] by scavenging ROS. For instance, supplementation of vitamin E to diabetic patients can decrease 7-KC and 7β-OH levels [75]. Likewise, Uemura *et al*. [76] found that apoptosis induced by 7β-OH and 7-KC in vascular endothelial cells was prevented by α-tocopherol. DHA exhibits potent anti-oxidant properties which attenuate ROS overproduction in endotelial cells [77] and enhances the overall antioxidant status [78]. Recently, the ability of DHA to prevent ROS overproduction and oxiapoptophagy induced by 7KC, 7β-OH, and 24-OH in oligodendrocytes was demonstrated [79]. De Medina *et al*. [80] found that DHA inhibited cholesterol-5,6-epoxide hydrolase activity, that catalyzes the conversion of 5,6α-E and 5,6β-E to its product CT, which exerts important citotoxic effects. Also, DHA showed protective effects on COPs-induced cell death [33]. These results agree with our findings, since the DHA-supplemented diet to orchidectomized rats prevented the rise in COPs formation caused by

orchidemtomy. Thus, the levels of COPs were near to those measured in control rats fed the control diet. Interestengly, the DHA-supplemented diet restored the increased contents of COPs induced by orchidectomy, similarly how it restores the lipid profile, and the redox and inflammatory status altered in this particular condition [9,10]. These results are in agreement with the cardioprotective effects of DHA widely reported through its anti-inflammatory [31] and anti-oxidant activities [33-35]. It has been recently reported that DHA, one of the main fatty acids of the Mediterranean diet, attenuates the 7-KC-induced toxic effect on microgial cells [81]. Based on all this information, human dietary habits deserve important consideration and comsumption of a Mediterranean diet could prevent the consequences of an increased oxidative stress that occurs in diverse physiopathological situations. However, something different seems to occur with the DHA-supplemented diet to the control animals, in which a tendency to increase COPs content was observed in the rat aorta. This result is in line with a previous report, in which the involvement of vasodilator prostanoids in the Ach-induced response in aorta from control rats was switched to vasoconstrictors after the DHA-suppelemented diet [9]. Overall, these results indicate that DHA-supplementation exerts cardiopotective effects, especially in pathological conditions, which seems to be reasonable since the homeostatic mechanisms work to maintain proper function in healthy subjects without any nutritional supplement.

In summary, we describe here the detrimental effects caused by the lack of sex hormones in the rise in lipid peroxidation, providing new information on the damage on lipid components responsible of maintaining membrane properties and cell signaling in different pathways involved in the vascular function of aorta and mesenteric artery, already published [9,10]. The preventive effect of a DHA-supplemented diet on lipid peroxidation, which promotes the maintenance of vascular homestasis, is also shown. In this regard, it is important to note that the results observed in the mesenteric artery, together with those related to vasomotor function [10], are of special relevance since this vascular bed importantly contributes to the control of the peripheral vascular resistance and therefore to the regulation of blood pressure. The present findings reveal the interest in conducting clinical studies with DHA in patients with decreased levels of sex hormones (elderly patients and/or prostate cancer patients undergoing androgen deprivation therapy) who display cardiovascular risk factors.

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Writing ± original draft: Diva M. Villalpando, Mercedes Ferrer.

Writing ± review & editing: Mibsam M. Rojas, Hugo S. García, Mercedes Ferrer.

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Conclusions / Conclusiones

Conclusions

In summary, the data obtained in the present study indicates that the DHA supplemented diet prevents vascular dysfunction produced by the loss of gonadal function in male rats, due to specific actions on:

1. **Lipid profile.** The DHA supplemented diet restored the lipid profile negatively affected by the orchidectomy.
2. **COPs**. The DHA supplemented diet prevented the orchidectomy-induced COPs increase.
3. **Redox status**. The DHA supplemented diet exerted antioxidant properties, since it decreased hydrogen peroxide formation and increased antioxidant capacity in both serum and aortic tissue. In addition DHA-supplemented diet decreased the production of superoxide anion (increased by orchidectomy) in aorta and mesenteric artery, which could account for the restored NO production and bioavailability in these arteries.
4. **Vascular inflammation**. DHA exerts anti-inflammatory properties by decreasing prostanoid production in both aorta and mesenteric artery.

The DHA induced effects here described could be involved in the maintencance of vascular function through: i) the improvement of the neurogenic response in mesenteric artery and ii) the participation of hyperpolarizing mechanisms in aorta, which contributes to preserve vascular function.

Overall, these results show that the DHA-supplemented diet exerts a cardioprotective effect in physiopathological conditions in which vascular dysfunction exists.

Conclusiones

En resumen, los datos obtenidos en el presente estudio indican que la dieta suplementada con DHA previene la disfunción vascular producida por la pérdida de la función gonadal en ratas macho, debido a acciones específicas sobre:

1. **El perfil lipídico**. La dieta suplementada con DHA restauró el perfil lipídico, afectado negativamente por la orquidectomía.
2. **Los productos de oxidación del colesterol**. La dieta suplementada con DHA previno el aumento de COPs inducido por la orquidectomía.
3. **El estado redox**. La dieta suplementada con DHA ejerció propiedades antioxidantes, ya que disminuyó la formación de peróxido de hidrógeno y aumentó la capacidad antioxidante tanto en el suero como en el tejido aórtico. Además, la dieta suplementada con DHA disminuyó la producción del anión superóxido (aumentada por la orquidectomía) en aorta y arteria mesentérica, lo que podría explicar la restauración en la producción y biodisponibilidad del NO en estas arterias.
4. **La inflamación vascular**. El DHA ejerce propiedades antiinflamatorias al disminuir la producción de prostanoides tanto en aorta como en arteria mesentérica.

Los efectos inducidos por el DHA aquí descritos podrían estar implicados en el mantenimiento de la función vascular a través de: i) la mejora de la respuesta neurogénica en la arteria mesentérica y ii) la participación de mecanismos hiperpolarizantes en la aorta, lo que contribuye a preservar la función vascular.

Globalmente, estos resultados muestran que una dieta suplementada con DHA ejerce un efecto cardioprotector en condiciones fisiopatológicas en las que existe disfunción vascular.